A Thesis for the Degree of Doctor of Philosophy

Application of Superheated Steam-Based Technology for Inactivation of Foodborne Pathogens

과열 수증기를 이용한 식품병원성균 제어 기술 연구

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Abstract

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Superheated steam (SHS) is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure. SHS has been known as a safe, non-polluting technology with low energy consumption and proven to be one of the most effective methods for the drying of biological or non-biological products, including foods. However, the inactivation of foodborne pathogens by SHS has rarely been studied. This study researched the effects of SHS treatment for inactivating foodborne pathogens including *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* by comparing and evaluating the effectiveness of SS and SHS.

Bacteria can attach to solid surfaces of food processing facilities and form slimy, slippery biofilms consisting of hydrated extracellular polymeric substances. Adhesion of bacteria to food processing facility surfaces leads to potential hygienic problems in the food processing industry. Biofilms were formed on PVC and stainless steel coupons by using a mixture of three strains each of three foodborne pathogens at 25℃. After biofilm development, PVC

and stainless steel coupons were treated with saturated steam (SS) at 100℃ and SHS at 125, 150, 175, and 200℃ for 5, 10, 20, and 30 s on both sides. The viable cell numbers of biofilms were significantly ($P < 0.05$) reduced as SHS temperature and exposure time increased. For all biofilm cells, SHS treatment resulted in an additional log reduction compared to SS treatments. After exposure to 200℃ steam for 30 s or 10 s on PVC or stainless steel, respectively, the numbers of biofilm cells were reduced to below the detection limit (1.48 log CFU/coupon). SHS treatment effectively reduced populations of biofilm cells and reduced disinfection time compared to SS treatments.

Effectiveness of SHS on the inactivation of foodborne pathogens on agriculture produce including almonds, in-shell pistachios, cherry tomatoes, oranges, radish seeds, and alfalfa seeds and on quality by measuring color, texture, ascorbic acid contents, antioxidant capacity, and germination rate were evaluated. Exposure of almonds and pistachios to SHS for 15 or 30 s at 200˚C reduced all tested pathogens to below the detection limit (0.3 log CFU/g) without causing significant changes in color values or texture parameters ($P > 0.05$). For both almonds and pistachios, acid and peroxide values following SS and SHS treatment for up to 15 s and 30 s, respectively, were within the acceptable range. Exposure to SHS for 3 or 20 s at 200˚C reduced all tested pathogens on cherry tomatoes and oranges, respectively, to below the detection limit (1 and 1.7 log CFU/g, respectively) without causing significant changes in color values or texture parameters, ascorbic acid contents, and antioxidant capacity ($P > 0.05$). SHS treatment caused to an

additional 0.79–2.05 and 0.78–1.77 log reductions of the three pathogens on radish seeds and alfalfa seeds treated continuous and intermittent (1 s heating followed by cooling at 25˚C for 2 min) steam treatment, respectively, compared to SS treatments. A continuous steam treatment for 3 and 2 s resulted in a considerably drop in percent germination compared to the water control for radish seeds and alfalfa seeds, respectively. However, 10 times intermittent SHS treatment at 200℃ did not decrease germination rate of radish seeds and alfalfa seeds under the 90%.

Simulation using computational fluid dynamics (CFD) was studied to evaluate the inactivation of foodborne pathogens on food samples by SHS treatment. COMSOL multi-physics software to predict temperature distribution and concentration of the live bacteria on an orange were used. The governing equations for continuity, compressible fluid flow, and energy are solved numerically together with bacteria concentration, using a finite element method. Arrhenius equation was used to describe bacteria deactivation kinetics. The simulations have provided flow pattern, live bacteria concentration, and temperature profiles from different periods of heating. The simulated results show the slowest heating and little effect zones, which are correlated to the concentration of the live bacteria. The simulations also show bacteria were eliminated during SHS treatment at 200℃ for 20 s.

Portable superheated steam generator for field application was developed and the ability of inactivation of foodborne pathogens biofilm cells on stainless steel evaluated. The populations of viable biofilm cells on stainless

steel coupons were reduced below the detection limit when subjected to SHS treatment at 160℃ for 30 s. Healthy cells and heat-injured cells on stainless steel coupons following SS or SHS heating were compared. There were no significant ($P > 0.05$) differences between the levels of cells enumerated on the appropriate selective agar (SMAC, XLD, and OAB) versus the agar for resuscitation (SPRAB, OV-XLD, and OV-OAB) during the whole SHS treatment time. Also, the results have revealed that the Weibull model, which had been mostly used for describing inactivation of the bacterial cells by heat treatment, could be successfully used to describe foodborne pathogens biofilm cells on stainless steel inactivation by SHS.

This study demonstrated that SHS treatment effectively reduced populations of biofilm cells on materials and foodborne pathogens on agricultural produce compared to SS treatments. And inactivation of bacteria on food during SHS treatment using CFD and development of portable SHS generator can be used for application to feeding facilities. SHS treatment has potential as an excellent intervention for controlling foodborne pathogens and enhancing safety in the food industry.

Key words: Superheated steam, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, Biofilm, Computational fluid dynamics, Portable

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General Introduction

1. Superheated steam

Superheated steam (SHS) is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure, and a drop in temperature of SHS will not result in condensation unless the temperature is decreased to below the saturation temperature point corresponding to the processing pressure (Cenkowski et al., 2007). It is first proposed in a German book as early as 1898, research interest in SHS drying seems to have begun about 50 years ago, and industrial developments started only about 35 years ago (Kumar and Mujumdar, 1990).

SHS as a drying medium for non-temperature sensitive products has many potential benefits to the industry and consumers and has been evaluated by many researchers (Van Deventer and Heijmans, 2001; Lane and Stern, 1956). Superheated steam system can lead to energy saving as high as 50 to 80% over use of hot air. The constant rate drying period is also longer in superheated steam drying (SSD), thus providing high drying rates for longer periods of time. Thus, higher drying rates will increase the efficiency of the processing operation, potentially leading to a reduction in equipment size or an increase in output (Pronyk and Cenkowski, 2004). High thermal efficiency is usually achieved only if the exhaust steam is collected and used elsewhere in the processing operation (Pronyk and Cenkowski, 2004). SHS as the drying

medium instead of hot air methods that there is an oxygen free environment. There is no oxidative or combustion reactions during drying (no fire or explosion hazards) and the oxygen free environment also produces improved product quality (Pronyk and Cenkowski, 2004). Most SHS dehydrators are designed as closed systems where the exhaust may be collected and condensed. In this way toxic or expensive compounds are removed and collected before they reach the environment, thus air pollution (Pronyk and Cenkowski, 2004). Disadvantages of the SHS technology are high capital cost, complexity of the equipment, and high temperature of processed products (important when processing temperature products) (Cenkowski et al., 2007). SHS has been investigated to dry products such as brewers' spent grain and distillers' spent grain (Tang and Cenkowski, 2005), potatoes (Tang and Cenkowski, 2000), potato chips (Caixeta et al., 2002), sugar beet pulp (Tang et al., 2000), wood pulp and paper (Douglas, 1994), Asian noodles (Markowski et al., 2003), lumber (Woods et al., 1994), coal (Potter and Beeby, 1994) and sludge (Francis and Di Bella, 1996).

SHS has long been known as a safe, non-polluting technology with low energy consumption (Chou and Chua, 2001). SHS transfers a larger amount of heat to the subject of treatment than SS (James et al., 2000; Topin and Tadrist, 1997). Several researchers have studied the effects of SHS for inactivating pathogens on chicken skin, raw almonds and other foods (Bari et al., 2010; Kondjoyan and Portanguen, 2008). Kondjoyan and Portanguen (2008) reported that SHS was clearly more efficient to inactivate *Listeria innocua*

than non-SHS methods, leading to an average reduction of more than 5 log after 30 s treatment. However, the inactivation of foodborne pathogens by SHS has rarely been studied.

2. Inactivation methods of foodborne pathogens

Foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* are a serious concern in food processing facilities. It is possible to occasionally find these pathogens in food processing facilities (Johansson et al., 1999; Hood and Zottola, 1997; Todd et al., 2009). *E. coli* O157:H7 is capable of causing bloody diarrhea and renal failure in humans (Doyle, 1991). *S.* Typhimurium causes diarrhea, fever, and abdominal cramps 12 to 72 hours after infection (Blaser and Newman, 1982). Listeriosis caused by *L. monocytogenes* results in meningitis, sepsis, encephalitis, febrile gastroenteritis, and abortion (Schlech, 2000).

Biofilms are structured bacterial communities enclosed with polymeric matrices of DNA, protein, and polysaccharides (Stoodley et al., 2002; Sutherland, 2001;Whitchurch et al., 2002), and protect bacterial cells against environmental stresses, detergents, antibiotics, and the host immune system (Bower and Daeschel, 1999; Costerton et al., 1999; Mah and O'Toole, 2001; Yasuda et al., 1994). Bacteria can attach to solid surfaces of food processing facilities (Wingender et al., 1999) and form slimy, slippery biofilms consisting of hydrated extracellular polymeric substances (Costerton et al., 1999). Adhesion of bacteria to food processing facility surfaces leads to potential hygienic problems in the food processing industry because the resultant surface adherent pathogenic biofilms transmit pathogens to food

(Barnes et al., 1999; Shi and Zhu, 2009). Bacteria can form biofilms on stainless steel, polyvinyl chloride (PVC), glass, and rubber (Pedersen, 1990; Prouty and Gunn, 2003; Ronner and Wong, 1993) and exhibit increased resistance to cleaning and disinfection compared to planktonic cells (Bower and Daeschel, 1999; Mah and O'Toole, 2001).

A lot of approaches have been carried out to inactivate biofilm cells, since conventional methods of controlling planktonic bacteria, including chemical detergents and physical treatments, often prove ineffective. Current procedures to remove biofilms include combinations of mechanical action, such as high pressure, and concurrent application of biocides (detergents (Gibson et al., 1999), matrix-hydrolyzing enzymes (Johansen et al., 1997), and oxidizing substances (Norwood and Gilmour, 2000)). The efficacy of biocides may be enhanced by the use of electric fields (Blenkinsopp et al., 1992) and ultrasound (Mott et al., 1998). However, these methods all have restrictions. High pressure spraying may spread live bacteria over the environment due to aerosol generation, and the use of oxidizing substances like chlorine may cause environmental pollution and pose health risks to humans. Furthermore, they are not applicable to high-throughput processing on a large-scale for the food industry.

In recent years, concerns about foodborne outbreaks involving low water activity (a_w) foods have increased (Scott et al., 2009), because salmonellosis is known to be linked to diverse dry foods such as almonds (Isaacs et al., 2005), peanuts, and peanut butter (CDC, 2009). Salmonellosis causes diarrhea, fever,

and abdominal cramps 12 to 72 h after infection (Baird-Parker, 1990; Blaser and Newman, 1982). More recently, *Escherichia coli* O157:H7 illnesses have been epidemiologically linked to consumption of in-shell hazelnuts (FDA, 2011). *E. coli* O157:H7 is a pathogen causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). In 2010 and 2014, walnuts were recalled after isolation of *Salmonella* (FDA, 2010) and *Listeria monocytogenes* (FDA, 2014). *L. monocytogenes* infection results in abortion, encephalitis, febrile gastroenteritis, meningitis, and sepsis (Schlech and Acheson, 2000). Cross contamination of raw almonds can readily occur under typical harvesting, drying, and hulling-shelling practices (CDC, 2004). Furthermore, foodborne pathogens are able to survive in dry environments such as almond kernels and pistachios for prolonged periods of time (Kimber et al., 2012; Uesugi et al., 2006).

To inactivate *Salmonella* on almonds, several methodologies such as propylene oxide fumigation (Danyluk et al., 2005), infrared heat (Brandl et al., 2008), hot oil (Du et al., 2010), high hydrostatic pressure (Willford et al., 2008), acidic sprays (Pao et al., 2006), chlorine dioxide (Wihodo et al., 2005), and steam (Chang et al., 2010; Lee et al., 2006) have been evaluated. However, a maximum residue limit of propylene oxide fumigant has not been established (Brandl et al., 2008) and chlorine dioxide can lead to discoloration of almond surfaces at high concentrations (Wihodo et al., 2005). In particular, saturated steam (SS) pasteurization increases moisture content of the nuts and thus, requires additional processing to remove excess moisture before storage

(Brandl et al., 2008).

Consumption of fresh produces has increased rapidly as consumers are becoming increasingly aware of health and nutrition (Heaton and Jones, 2008). Concomitant with increased consumption of fresh produce come increasing frequency of foodborne disease outbreaks (Sivapalasingam et al., 2004). Fresh produce can become contaminated with foodborne pathogens while growing in fields, orchard, vineyards, or greenhouses, or during harvesting, post-harvest handling, and processing (Beuchat, 1996; 2002). For this reason, controlling foodborne pathogens on fruits and vegetables becomes important to ensure microbial safety and promote consumer health.

To sanitize fresh produce, washing with chlorinated water has widely been used on a commercial scale to reduce the microbial load (Parish et al., 2003; Weissinger et al., 2000). However, this treatment produces an antimicrobial effect of less than 2 log CFU/g on fresh fruits and vegetables (Beuchat, 1999; Taormina and Beuchat, 1999) and is known to adversely react with organic matter, resulting in the formation of carcinogenic halogenated by-products (Hua and Reckhow, 2007). Furthermore, continuous exposure to chlorine-based sanitizers has the effect of increasing resistance of microorganisms (Davidson and Harrison, 2002). Furthermore, consumers prefer that fresh produce not be treated with chemicals. Therefore, an alternative new method is needed to effectively reduce pathogens and simultaneously reduce or eliminate chemical use while still maintaining quality.

In recent years, consumption of vegetable sprouts has increased rapidly as

consumers are becoming increasingly health awareness. These sprouts need no preparation and provide availability and high nutritive value including vitamins, minerals, etc. (Meyerowitz, 1999, Weiss and Hammes, 2003). For more than a decade, accordingly, there has been a growth in the frequency of outbreak linked to the consumption of raw sprouts. In 2009 and 2010, a multistate outbreak of *Salmonella* infection, eventually with 228 and 184 cases, was associated with alfalfa sprouts produced at multiple facilities from seeds that likely originated from a common cultivator, respectively (CDC, 2010; Safranek, 2009). The causative bacteria of sprout-related outbreaks were *Escherichia coli* O157:H7 and *Salmonella* spp. (Stewart, 2001a; Stewart, 2001b) and *Listeria monocytogenes* which has been isolated from commercially produced sprouted seeds, but no cases of human listeriosis have been linked to those sprouts (National Advisory Committee on Microbiological Criteria for Foods, 1999).

To inactivate foodborne pathogens on sprout seeds, various methods such as hot water treatment (Bari et al., 2008), chermical treatments (Taormina and Beuchat, 1999a; Weissinger and Beuchat, 2000), gamma irradiation (Thayer et al., 2003), high hydrostatic pressure (Neetoo et al., 2008), ozone (Sharma et al., 2003; Wade et al., 2003), ultrasound (Scouten and Beuchat, 2002) have been evaluated. However, treatment with 20,000 ppm chlorine failed to eliminate the pathogen on seeds containing 2.7 log CFU/g (Taormina and Beuchat, 1999a). Chemical treatment has little antimicrobial effect of less than 2 log CFU/g on seeds (Taormina and Beuchat, 1999a).

3. Computational fluid dynamics for food industry

Computational fluid dynamics (CFD) was originally developed from the pioneering accomplishments of enthusiasts who in their endeavors to obtain insight into fluid motion instigated the development of powerful numerical techniques that have advanced the numerical methods of all types of fluid flow (Shang, 2004). CFD is a simulation tool, which uses powerful computers in combined with applied numerical methods to model fluid flow situations and purpose in the optimal new design of manufacture (Kuriakose and Anandharamakrishnan, 2010).

Although the origin of CFD can be found in the aerospace, automotive, and nuclear industries, it is only in the recent years that CFD has been applied to the food processing (Scott, 1994). CFD application in the food industry would support in a better understanding of the complex physical mechanisms that govern the physical, thermal, and rheological properties of food (Xia and Sun, 2002). Several researchers such as Scott and Richardson (1997), Quarini (1995) have researched the general application of CFD to the food processing industry and specific areas such as static mixers (Scott, 1977), clean-room design, refrigerated transport (Janes and Dalgly, 1996), and pipe flow (Scott, 1996).

The CFD provides a detailed understanding of flow distribution, weight losses, mass and heat transfer, particulate separation. Therefore, plant manager can take a much better and deeper understanding of what is

happening in a particular process or system. CFD makes possible to evaluate geometric changes with much less time and cost than would be involved in laboratory scale testing. It can reduce scale-up problems because the models are based on fundamental physics and are scale independent (Wanot, 1996).

Recently, there has been enormous development of commercial CFD codes to enhance their combination with the sophisticated modelling requirements of many research areas, thereby emphasizing their versatility and attractiveness (Norton and Sun, 2006). Among the many codes that exist today not all provide the features required by the food engineer (Norton and Sun, 2006). Such requirements include the provision of powerful preprocessor, solver and post-processor environments, the power to import grid geometry, initial conditions, and boundary conditions from an external text file, flow dependent properties, phase change and flow through porous media (Kopyt and Gwarek, 2004). The commercial software packages such as CFX, FLUENT, PHOENICS, and COMSOL featured these functionalities, employ graphical user interfaces, and support Windows, UNIX and Linux platforms.

Objectives of this study

Superheated steam has been proven to be one of the most effective methods for the drying of biological or non-biological products, including foods. However, the inactivation of foodborne pathogens by superheated steam has rarely been studied.

Furthermore, to ensure both food preservation and safety, it is necessary to promote quality characteristics of food while eradicating the threat of spoilage. For this to occur efficiently, the appropriate temperature and treatment duration need to be known based on the physical process, from the analysis of measured data or physical properties, over a range of experimental conditions. Therefore, this study was performed to evaluate the effectiveness of SHS for inactivating foodborne pathogens, which include as follows:

1. **Inactivation of foodborne pathogens biofilm cells formed by attachment on food processing facility materials by superheated steam.**
2. **Effects of superheated steam to inactivate foodborne pathogens on agricultural produce.**
3. **Computational fluid dynamics in design and analysis of superheated steam treatments.**
4. **Development of portable superheated steam generator and inactivation kinetics of foodborne pathogens biofilm cells.**

Chapter I

Inactivation of Foodborne Pathogens Biofilm Cells on Materials used in Food Processing Facilities

Chapter I(1)

Effect of Chlorine, Hydrogen Peroxide, Quaternary Ammonium, and Iodophor Combined with Steam Heating on the Inactivation of Foodborne Pathogens in a Biofilm on Stainless Steel

I(1)-1. Introduction

Escherichia coli O157:H7, *Salmonella* Typhimurium, *and Listeria monocytogenes* are the main pathogens implicated in numerous foodborne outbreaks. *E. coli* O157:H7 is an important pathogen capable of causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). Salmonellosis caused by *Salmonella* results in diarrhea, abdominal pain, fever, chills, nausea, and vomiting (Baird-Parker, 1990). The principal symptoms of *L. monocytogenes* infection are abortion, neonatal death, septicemia, and meningitis (Farber and Peterkin, 1991). Contamination with these pathogens in food-processing environments and food-processing lines may be a frequent and important cause of outbreaks of food-borne disease (Reij and Den Aantrekker, 2004).

Improper cleaning and disinfection of food contact surfaces contributes to soil buildup, and, in the presence of water, facilitates the development of bacterial biofilms which may include pathogenic microorganisms (Chmielewski and Frank, 2003). A biofilm is a sessile bacterial community of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in extracellular polymeric substances (Costerton, 1995; Donlan, 2002). Such biofilms can be a continuous source of contamination to foods coming in contact with them when processed on contact surfaces.

Many researchers have studied the effectiveness of sanitizers used in the food industry against food-borne pathogens, including chlorine and chlorine derivatives, iodophors, quaternary ammonium compounds (QAC) and hydrogen peroxide (HP) (Greene et al., 1993; Peng et al., 2002; Joseph, et al., 2001). However, individual sanitizer treatments used in many studies showed little effect on cells in a biofilm even when long time exposure times were utilized (Joseph et al., 2001; Chmielewski and Frank, 2003). Furthermore, bacteria within a biofilm matrix have decreased sensitivity to disinfectants compared to planktonic cells, and the resistance of biofilm bacteria typically increases with age (Bower et al., 1996; Costerton et al., 1999)

To improve the ability of killing and removing biofilm organisms from food processing facilities, combination treatments of sanitizers and other methods are required. Combination treatments are utilized because it is expected that the use of combined factors will have greater effectiveness at inactivating microorganisms than the use of any single factor alone. Many researchers have evaluated combinations of sanitizers with other cleaning methods such as, ultrasonication, heat, and other sanitizers to eradicate or inhibit foodborne pathogens (Berrang et al., 2008; Scouten and Beuchat, 2002; Jin and Lee, 2007).

Steam treatment is a rapid method of heating that has previously been studied for inactivating foodborne pathogens on foods and biofilms in food processing environments (Chang et al., 2010; Ban et al., 2012). The main advantage of steam treatment is the large amount of heat transferred to the

food or material when steam condenses, which rapidly increases surface temperature (James et al., 2000). Steam at 100℃ has a greater heat capacity than the same amount of water at that temperature (James and James, 1997) In addition, steam is able to penetrate cavities, scale follicles while water cannot reach all the contaminated surfaces because of the high surface tension of aqueous fluids (Morgan et al., 1996). However, to date, no studies have investigated the combination of steam and sanitizers such as sodium hypochlorite (SHC), iodophor, benzalkonium chloride (BKC; kind of QAC), and HP for reducing biofilms.

Therefore, the objective of this study was to determine and compare the effectiveness of individual treatments (steam and sanitizers) and the combination of steam and sanitizers for reducing foodborne pathogenic biofilm cells on stainless steel.

I(1)-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at –80℃ in 0.7 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. Working cultures were maintained on tryptic soy agar (TSA; Difco) slants at 4℃ and were subcultured monthly. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37℃ for 24 h. Culture cocktails of each pathogen species were prepared individually as follows: the three strains of each pathogen species were combined and cells were collected by centrifugation at 5000 g at 4℃ for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final pellets of each pathogen species were resuspended in sterile PBS, corresponding to approximately 10^7–10^8 colony-forming units (CFU)/ml.

2.2. Preparation of stainless steel coupons

Stainless steel coupons (type 316, 5 × 2 × 0.1 cm^3, bright annealed) were used in this study. Coupons were immersed in 70% ethanol for 10 min to

disinfect the surface, and rinsed with sterile distilled water. Washed stainless steel coupons were autoclaved at 121℃ for 15 min in covered glass beakers before use.

2.3. Biofilm formation

Prepared sterile stainless steel coupons were transferred to sterile 50 ml conical centrifuge tubes (SPL Lifesciences, Pocheon, Korea) containing 30 ml of a single pathogen species culture cocktail (*E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes*) cell suspension in PBS (ca. 10^7–10^8 CFU/ml). Conical centrifuge tubes containing coupons were incubated at 4℃ for 24 h to facilitate attachment of cells. After incubation, coupons were aseptically removed with sterile forceps, immersed in 300 ml of sterile distilled water (22 ± 2℃), and gently stirred for 5 s. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of TSB, and then incubated at 25℃ for 6 days. This method was adapted from Kim et al. (2006).

2.4. Sanitizer Preparation

The sanitizers tested were SHC (Yuhan Co., Incheon, Korea), HP (Junsei Chemical Co., Tokyo, Japan), BKC (3M, USA), and iodophor (Namkang Co., Incheon, Korea). The sanitizers were all diluted according to manufacturers' instructions with sterile distilled water to the target concentration; The concentration of free chlorine was quantitated using a HI 95771 Chlorine

Ultra High Range Meter (Hanna Instruments, Ann Arbor, MI, USA). The solutions were prepared on the day experiments were performed.

2.5. Combination treatment of sanitizer and steam

Coupons were removed with sterile forceps, rinsed for 5 s in 300 ml of sterile distilled water (22 ± 2℃), then immersed in each type of sanitizer for 5, 15, and 30 s. Then they were treated with steam on both sides for 5, 10, and 20 s, respectively, while maintaining an absolute pressure of 143kPa. During these experiments, the distance between the coupons and the steam outlet was set at 40 mm. Coupons treated with steam alone or immersed into each sanitizer alone were used as controls.

2.6. Bacterial enumeration

After treatment, stainless steel coupons were transferred to sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Immediately after vortexing, cell suspensions were tenfold serially diluted in buffered peptone water (BPW; Difco), and 0.1 ml of undiluted cell suspension or dilutions were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), or Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate the number of *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* biofilm cells, respectively, attached to

the surfaces of stainless steel coupons. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective medium. The plates were incubated at 37℃ for 24–48 h. After incubation, colonies were counted. The detection limit for three pathogens by direct plating was 1.48 log CFU/coupon.

2.7. Confocal laser scanning microscopy

In order to examine cell membrane integrity, a BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was used. This kit includes SYTO9 and propidium iodide (PI) to differentiate between cells with intact membranes (live) and damaged membranes (dead), respectively. Viable cells appeared green in color, whereas damaged cells were stained red. The stain was prepared by diluting 3 μl of each component into 1 ml of distilled water. For confocal laser scanning microscopy (CLSM, Eclipse 90i, Nikon, Japan), biofilm coupons treated with sanitizer and steam were stained with 0.1 ml of each staining solution for 30 min in the dark. Biofilm samples were photographed with an upright CLSM using a 60X water immersion objective lens with a numerical aperture of 0.9. Image stacks at various foci collected through the CLSM were reconstructed in three-dimension using IMARIS software (Bitplane, Zurich, Switzerland) (Park et al., 2011).

2.8. Statistical analysis

All experiments were repeated three times with duplicate samples. Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple range test at a probability level of $P < 0.05$.

I(1)-3. Results

3.1. Inactivation of E. coli O157:H7 biofilm on stainless steel

Table 1 shows numbers of surviving CFU in biofilm formed by *E. coli* O157:H7 on stainless steel enumerated on SMAC agar after each sanitizer and steam treatment. The initial level of *E. coli* O157:H7 biofilm on stainless steel was 6.26 log CFU/coupon. Slight reductions (< 0.5 log) occurred when inoculated coupons were rinsed in distilled water.

The numbers of surviving *E. coli* O157:H7 biofilm cells were significantly ($P < 0.05$) reduced as the concentration of sanitizer and duration of steam treatment increased.

Stainless steel coupons immersed in SHC alone or exposed to steam treatment alone experienced a log reduction range of 0.16–1.12 or 0.67–2.5 for *E. coli* O157:H7 biofilm cells, respectively, compared to that of the distilled water control. *E. coli* O157:H7 biofilm cells were reduced by 1.33–4.66 log after the combination treatment of SHC and steam. A synergistic effect was observed such that the combination treatment achieved an additional 0.04–2.33 log reduction compared to the sum of the individual treatments. However, the synergy effect was very slight or not observed when coupons were dipped in 20 ppm SHC. Biofilm cells on stainless steel coupons were reduced to below the detection limit (< 1.48 log) when immersed in 100 ppm SHC for 15 or 30 s and then steamed for 10 s, or when immersed in 50

ppm SHC for 30 s or 100 ppm for 5, 15, or 30 s and then steam treated for 20 s.

The results of the combination treatment of steam and iodophor, BKC, or HP on stainless steel coupons showed a similar tendency.

Table I(1)-1. Survival (log CFU/coupon) of *E. coli* O157:H7 in biofilm formed on the surface of stainless steel coupons treated with steam and sodium hypochlorite, hydrogen peroxide, benzalkonium chloride, and iodophor

Con. (ppm)	Immer-sion (s)	Sodium hypochlorite Treatment time (s) 0	5	10	20
0	-	6.14±0.05 Aa	5.47±0.07 Ab	4.79±0.15 Ac	3.64±0.17 Ad
20	5	5.98±0.07 ABa	5.27±0.10 ABb	4.62±0.07 Ac	3.56±0.23 ABd
	15	5.90±0.05 ABa	5.17±0.15 ABb	4.57±0.09 Ac	3.33±0.56 ABd
	30	5.59±0.03 Ca	4.97±0.12 Bb	3.93±0.07 Bc	2.11±0.24 Dd
50	5	5.84±0.26 Ba	4.92±0.30 Bb	3.70±0.19 Cc	3.18±0.14 Bd
	15	5.40±0.32 CDa	3.71±0.41 Cb	3.62±0.08 Cb	2.52±0.18 Cc
	30	5.17±0.10 DEa	3.47±0.28 Cb	2.33±0.23 Ec	< 1.48 Ed
100	5	5.54±0.11 Ca	3.72±0.33 Cb	2.73±0.19 Dc	< 1.48 Ed
	15	5.16±0.08 DEa	2.83±0.24 Db	< 1.48 Fc	< 1.48 Ec
	30	5.02±0.04 Ea	2.35±0.13 Eb	< 1.48 Fc	< 1.48 Ec
Con. (%)	Immer-sion (s)	Hydrogen peroxide Treatment time (s) 0	5	10	20
0	-	6.14±0.05 Aa	5.47±0.07 Ab	4.79±0.15 Ac	3.64±0.17 Ad
0.5	5	6.03±0.17 ABa	4.77±0.12 Bb	4.12±0.04 Bc	3.33±0.19 Bd
	15	5.71±0.32 ABCa	4.21±0.12 Cb	3.28±0.06 Cc	2.26±0.10 Dd
	30	5.49±0.37 BCDa	3.46±0.04 DEb	2.86±0.14 Dc	1.97±0.12 Ed
1	5	5.79±0.37 ABCa	4.20±0.17 Cb	3.23±0.10 Cc	2.66±0.29 Cd
	15	5.45±0.29 CDa	3.81±0.18 CDb	3.12±0.04 Cc	2.15±0.09 DEd
	30	5.06±0.48 Da	3.03±0.38 FGb	2.24±0.08 Ec	< 1.48 Fd
2	5	5.48±0.16 BCDa	3.47±0.24 DEb	2.79±0.37 Dc	< 1.48 Fd
	15	5.07±0.33 Da	3.30±0.27 EFb	< 1.48 Fc	< 1.48 Fc
	30	4.17±0.12 Ea	< 1.48 Gb	< 1.48 Fc	< 1.48 Fc

		Benzalkonium chloride			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.14±0.05 A a	5.47±0.07 Ab	4.79±0.15 Ac	3.64±0.17 Ad
20	5	6.02±0.03 ABa	5.35±0.11 ABb	4.70±0.17 Ac	3.48±0.21 ABd
	15	5.85±0.07 ABCDa	5.23±0.12 ABCb	4.52±0.13 ABc	3.25±0.29 BCd
	30	5.54±0.25 DEFa	5.05±0.03 CDb	4.29±0.11 BCc	3.16±0.25 Cd
50	5	5.92±0.06 ABCa	5.33±0.06 ABb	4.50±0.30 ABc	3.23±0.05 BCd
	15	5.71±0.16 BCDEa	5.19±0.15 BCb	4.27±0.26 BCc	3.01±0.21 Cd
	30	5.45±0.23 EFa	4.88±0.04 Db	4.00±0.07 Cc	2.43±0.11 Dd
100	5	5.74±0.11 BCDEa	4.08±0.22 Eb	3.18±0.17 Dc	< 1.48 Ed
	15	5.67±0.14 CDEa	3.79±0.26 Fb	2.76±0.05 Ec	< 1.48 Ed
	30	5.24±0.34 Fa	2.53±0.15 Gb	< 1.48 Fc	< 1.48 Ec
		Iodophor			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.14±0.05 Aa	5.47±0.07 Ab	4.79±0.15 Ac	3.64±0.17 Ad
0.5	5	5.97±0.15 ABa	5.32±0.04 Ab	4.63±0.16 ABc	3.49±0.21 Ad
	15	5.74±0.14 BCa	4.74±0.15 Bb	4.32±0.25 Bc	3.12±0.23 Bd
	30	5.30±0.21 Da	4.28±0.28 Cb	3.27±0.33 Cc	< 1.48 Dd
1	5	5.96±0.20 ABCa	4.78±0.18 Bb	4.31±0.22 Bc	3.36±0.33 ABd
	15	5.24±0.17 Da	4.63±0.18 BCb	3.48±0.28 Cc	1.97±0.35 Cd
	30	4.65±0.20 Ea	3.47±0.34 Db	2.02±0.31 Ec	< 1.48 Dd
2	5	5.67±0.10 Ca	4.40±0.26 BCb	3.36±0.32 Cc	< 1.48 Dd
	15	4.74±0.24 Ea	3.10±0.21 Eb	2.48±0.31 Dc	< 1.48 Dd
	30	4.27±0.06 Fa	2.71±0.17 Fb	< 1.48 Fc	< 1.48 Dc

Data represent means ± standard deviations. Means with the same uppercase letter in the same column are not significantly different ($P < 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.2. Inactivation of S. Typhimurium *biofilms on stainless steel*

Table 2 shows survival of *S.* Typhimurium biofilm cells after each sanitizer and steam treatment. The initial population of *S.* Typhimurium biofilm cells on stainless steel was 6.43 log CFU/coupon.

S. Typhimurium biofilm cell populations on stainless steel coupons experienced a log reduction range of 0.46–2.2 after treatment with SHC alone, compared to the distilled water control. Reduction levels of *S.* Typhimurium biofilm cells were 0.82–2.25 log after steam alone and 1.03–4.85 log after combination treatment of steam and SHC. The combination treatment resulted in an additional 0.02–1.83 log reduction compared to the sum of the individual treatments when coupons were immersed in 100 ppm SHC and then subjected to steam treatment. The numbers of viable biofilm cells on stainless steel were reduced to below the detection limit (< 1.48 log) when immersed in 100 ppm SHC for 15 s and then steamed for 10 or 20 s, or 100 ppm SHC for 30 s and then steamed for 5, 10 or 20 s.

The combination treatments of steam and BKC or iodophor were more effective than the combination treatment of steam and SHC for inactivating of *S.* Typhimurium biofilm cells on stainless steel coupons, though these sanitizers were at lower concentrations compared to SHC. Furthermore, populations of *S.* Typhimurium biofilm cells treated with steam and iodophor achieved an additional 0.01–2.3 log reduction compared to the sum of the individual treatments.

Table I(1)-2. Survival (log CFU/coupon) of *S.* Typhimurium in biofilm formed on the surface of stainless steel coupons treated with steam and sodium hypochlorite, hydrogen peroxide, benzalkonium chloride, and iodophor

		Sodium hypochlorite			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.33±0.06 Aa	5.51±0.09 Ab	4.80±0.15 Ac	4.08±0.22 Ad
20	5	5.87±0.08 Ba	5.30±0.21 Bb	4.70±0.19 ABc	3.89±0.13 Ad
	15	5.46±0.33 BCDa	5.12±0.13 BCa	4.38±0.28 Bb	3.31±0.24 Bc
	30	5.13±0.45 DEa	4.69±0.08 Ea	3.92±0.11 Cb	2.80±0.13 Cc
50	5	5.62±0.11 BCa	4.96±0.03 CDb	4.53±0.26 ABc	3.47±0.14 Bd
	15	5.27±0.16 CDEa	4.82±0.12 DEb	4.36±0.14 Bc	3.25±0.31 Bd
	30	4.86±0.09 EFa	4.05±0.06 Fb	3.73±0.24 Cb	2.59±0.38 Cc
100	5	5.14±0.29 DEa	3.66±0.05Gb	2.65±0.29 Dc	2.04±0.18 Dd
	15	4.54±0.32 Fa	2.79±0.20 Hb	< 1.48 Ec	< 1.48 Ec
	30	4.13±0.15 Ga	< 1.48 Ib	< 1.48 Eb	< 1.48 Eb
		Hydrogen peroxide			
		Treatment time (s)			
Con. (%)	Immer-sion (s)	0	5	10	20
0	-	6.33±0.06 A a	5.51± 0.09 Ab	4.80±0.15 Ac	4.08±0.22 Ad
0.5	5	5.83±0.15 Ba	4.77±0.14 Bb	4.26±0.12 Bc	3.82±0.27 Bd
	15	5.57±0.19 BCa	4.33±0.27 CDb	3.38±0.09 CDc	2.89±0.14 Cd
	30	5.46±0.14 BCa	3.80±0.19 Eb	3.10±0.41 Dc	< 1.48 Ed
1	5	5.69±0.10 BCa	4.53±0.06 BCb	3.49±0.14 Cc	2.89±0.04 Cd
	15	5.31±0.21 CDa	4.08±0.19 DEb	2.69±0.27 Ec	2.21±0.12 Dd
	30	4.98±0.19 Da	3.31±0.20 Fb	2.07±0.11 Fc	< 1.48 Ed
2	5	5.54±0.17 BCa	3.88±0.04 Eb	2.21±0.19 Fc	< 1.48 Ed
	15	5.07±0.18 Da	3.32±0.51 Fb	< 1.48 Gc	< 1.48 Ec
	30	4.57±0.44 Ea	< 1.48 Gb	< 1.48 Gc	< 1.48 Ec

		Benzalkonium chloride			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.33±0.06 A a	5.51± 0.09 Ab	4.80±0.15 Ac	4.08±0.22 Ad
20	5	6.17±0.09 ABa	5.43±0.08 ABb	4.64±0.08 ABc	4.03±0.22 Ad
	15	5.97±0.02 BCa	5.20±0.11 BCb	4.40±0.14 BCc	3.88±0.06 Ad
	30	5.79±0.10 CDa	4.83±0.14 Db	3.76±0.10 Dc	3.29±0.14 Bd
50	5	5.88±0.15 CDa	5.13±0.09 Cb	4.38±0.18 BCc	3.04±0.05 Cd
	15	5.67±0.19 Da	4.86±0.12 Db	3.72±0.31 Cc	2.63±0.16 Dd
	30	5.29±0.26 Ea	4.04±0.09 Eb	2.28±0.11 Dc	< 1.48 Ed
100	5	5.77±0.21 CDa	4.84±0.03 Eb	3.17±0.30 Ec	< 1.48 Ed
	15	5.22±0.16 Ea	3.46±0.35 Fb	2.64±0.30 Fc	< 1.48 Ed
	30	4.94±0.05 Fa	2.91±0.15 Gb	< 1.48 Gc	< 1.48 Ec
		Iodophor			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.33±0.06 A a	5.51± 0.09 Ab	4.80±0.15 Ac	4.08±0.22 Ad
0.5	5	6.17±0.18 ABa	5.27±0.14 Ab	4.63±0.13 Ac	3.71±0.32 Bd
	15	5.91±0.14 BCa	4.81±0.18 Bb	3.73±0.18 Bc	2.69±0.22 Cd
	30	5.56±0.18 CDa	4.49±0.27 Bb	3.22±0.39 CDc	< 1.48 Dd
1	5	5.66±0.22 CDa	4.76±0.07 Bb	3.74±0.12 Bc	2.60±0.11 Cd
	15	5.56±0.14 CDa	3.64±0.16 Cb	3.29±0.24 Cb	2.54±0.23 Cc
	30	5.13±0.18 EFa	3.33±0.29 CDb	2.92±0.03 Dc	< 1.48 Dd
2	5	5.38±0.35 DEa	3.13±0.28 Db	2.31±0.03 Ec	< 1.48 Dd
	15	5.00±0.24 Fa	2.51±0.15 Eb	< 1.48 Fc	< 1.48 Dc
	30	4.60±0.24 Ga	< 1.48 Fb	< 1.48 Fb	< 1.48 Db

Data represent means ± standard deviations. Means with the same uppercase letter in the same column are not significantly different ($P < 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.3. Inactivation of L. monocytogenes biofilms on stainless steel

Table 3 shows survival of *L. monocytogenes* biofilm cells after sanitizer and steam treatments. The initial population of *L. monocytogenes* biofilm cells was 6.38 log CFU/coupon.

The numbers of *L. monocytogenes* biofilm cells on stainless coupons were reduced by 0.3–1.34 log in SHC alone. Cell numbers were reduced by 0.52–2.16 log in steam alone and 0.76–4.64 log following the combined treatment of SHC and steam. The combination treatment achieved an additional 0.01–2.78 log reduction compared to the sum of the individual treatments. Levels of biofilm cells on stainless steel were reduced to below the detection limit (< 1.48 log) when submerged in 50 ppm SHC for 30 s and then steam treated for 30s; when immersed in 100 ppm SHC for 5 s and then steamed for 30 s; when immersed in 100 ppm SHC for 10 s and then steamed for 10 or 20 s; or when submerged in 100 ppm SHC for 30 s and then steamed for 5, 10 or 20 s.

A similar tendency was observed for combinations of steam and HP, BKC, or iodophor on stainless steel coupons. Populations of *L. monocytogenes* biofilm cells on stainless steel were reduced more when SHC and steam were used in combination compared to combinations of steam and the other sanitizers.

Table I(1)-3. Survival (log CFU/coupon) of *L. monocytogenes* in biofilm formed on the surface of stainless steel coupons treated with steam and sodium hypochlorite, hydrogen peroxide, benzalkonium chloride, and iodophor

		Sodium hypochlorite			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.12±0.16 Aa	5.60±0.12 Ab	4.81±0.04 Ac	3.96±0.07 Ad
20	5	5.82±0.11 Ba	5.26±0.06 ABb	4.70±0.11 Ac	3.91±0.09 ABd
	15	5.78±0.08 Ba	4.89±0.28 BCb	4.31±0.09 Bc	3.40±0.16 BCd
	30	5.68±0.08 BCa	4.60±0.37 CDb	3.71±0.12 Cc	3.02±0.22 Cd
50	5	5.73±0.08 Ba	4.71±0.21 Cb	4.48±0.07 ABb	3.71±0.28 ABc
	15	5.30±0.07 Da	4.16±0.24 DEb	3.82±0.08 Cc	3.11±0.08 Cd
	30	5.06±0.18 Ea	3.82±0.41 Eb	3.57±0.24 CDb	< 1.48 Dc
100	5	5.51±0.19 Ca	4.41±0.29 CDb	3.33±0.08 Dc	< 1.48 Dd
	15	4.91±0.07 EFa	2.94±0.21 Fb	< 1.48 Ec	< 1.48 Dc
	30	4.78±0.05 Fa	< 1.48 Fb	< 1.48 Ec	< 1.48 Dc
		Hydrogen peroxide			
		Treatment time (s)			
Con. (%)	Immer-sion (s)	0	5	10	20
0	-	6.12±0.16 A a	5.60±0.12 Ab	4.81±0.04 Ab	3.96±0.07 Ac
0.5	5	5.82±0.15 ABa	4.82±0.22 Bb	4.45±0.38 Ac	3.26±0.15 Bd
	15	5.61±0.20 BCa	4.50±0.16 BCb	3.86±0.19 Bc	2.87±0.33 Cd
	30	5.41±0.28 CDa	4.18±0.24 CDb	3.33±0.14 BCc	< 1.48 Dd
1	5	5.63±0.16 BCa	4.41±0.15 Cb	3.33±0.34 BCc	2.69±0.38 Cd
	15	5.43±0.17 CDa	3.82±0.24 DEFb	3.25±0.08 BCc	2.59±0.28 Cd
	30	5.12±0.15 DEa	3.59±0.38 FGb	2.50±0.49 Dc	< 1.48 Dd
2	5	5.32±0.28 CDa	4.01±0.07 DEb	3.20±0.51 Cc	< 1.48 Dd
	15	5.11±0.19 DEa	3.70±0.03 EFGb	2.76±0.47 CDc	< 1.48 Dd
	30	4.95±0.15 Ea	3.32±0.30 Gb	2.22±0.41 Dc	< 1.48 Dd

		Benzalkonium chloride			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.12±0.16 Aa	5.60±0.12 Ab	4.81±0.04 Ac	3.96±0.07 Ad
20	5	6.00±0.05 Aa	5.43±0.12 Ab	4.62±0.04 ABc	3.84±0.14 A d
	15	5.88±0.08 ABa	5.06±0.11 Bb	4.39±0.10 Bc	3.36±0.12 Bd
	30	5.70±0.03 Ca	4.65±0.08 Cb	3.87±0.19 Cc	2.91±0.15 Cd
50	5	5.93±0.07 ABa	5.00±0.05 Bb	4.41±0.11 Bc	3.42±0.13 Bd
	15	5.73±0.12 BCa	4.58±0.19 Cb	3.60±0.14 Dc	3.00±0.08 Cd
	30	5.54±0.16 CDa	4.17±0.15 Db	2.63±0.17 Fc	2.36±0.16 Dc
100	5	5.84±0.07 Ba	4.42±0.10 CDb	3.25±0.27 Ec	< 1.48 Ed
	15	5.53±0.04 Da	3.56±0.28 Eb	< 1.48 Gc	< 1.48 Ec
	30	5.40±0.11 Da	2.66±0.19 Fb	< 1.48 Gc	< 1.48 Ec
		Iodophor			
		Treatment time (s)			
Con. (ppm)	Immer-sion (s)	0	5	10	20
0	-	6.12±0.16 Aa	5.60±0.12 Aa	4.81±0.04 Ab	3.96±0.07 Ac
0.5	5	5.87±0.13 Ba	5.32±0.06 Bb	4.55±0.25 Ac	3.77±0.14 Ad
	15	5.77±0.22 BCa	4.92±0.04 Cb	3.75±0.28 BCc	3.08±0.28 Bd
	30	5.48±0.11 DEa	4.73±0.09 CDb	3.29±0.37 CDc	2.45±0.38 Cd
1	5	5.55±0.06 CDa	4.79±0.07 Cb	3.87±0.22 Bc	2.95±0.11 Bd
	15	5.15±0.04 GFa	4.50±0.23 Eb	3.38±0.26 BCDc	2.48±0.34 Cd
	30	4.92±0.22 GHa	3.88±0.15 Fb	2.49±0.39 Ec	< 1.48 Dd
2	5	5.31±0.34 EFa	4.56±0.10 DEb	3.12±0.52 Dc	< 1.48 Dd
	15	4.99±0.42 Ga	3.42±0.17 Gb	2.35±0.37 Ec	< 1.48 Dc
	30	4.72±0.10 Ha	3.05±0.08 Hb	< 1.48 Fc	< 1.48 Dc

Data represent means ± standard deviations. Means with the same uppercase letter in the same column are not significantly different ($P < 0.05$). Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.4. Effect of sanitizer and steam treatment on membrane integrity

CLSM images were recorded to analyze the morphologies of *E. coli* O157:H7 biofilms following each sanitizer and steam treatment. Fig. 1 shows that destruction of the cell membrane in *E. coli* O157:H7 biofilms was dependent on the concentration and property of sanitizer and steam duration. CLSM images of *E. coli* O157:H7 biofilms were captured after treatment with the water control; steam for 20 s; 100 ppm SHC for 30 s; 100 ppm SHC for 30 s and steam for 20 s; 2% HP for 30 s; 2% HP for 30 s and steam for 20 s; 100 ppm BKC for 30 s; 100 ppm BKC for 30 s and steam for 20 s; 100 ppm iodophor for 30 s; and 100 ppm iodophor for 30 s and steam for 20 s. The green and red stained cells indicate those having intact and damaged cell membranes (i.e., live and damaged/dead cells), respectively. When *E. coli* O157:H7 biofilm was treated with distilled water without steam and sanitizer exposure, almost all of the cells remained intact, as shown in Fig. 1a. *E. coli* O157:H7 biofilm cells after individual treatments were stained mostly green, with a few red cells being present, as shown in Fig. 1b, 1c, 1e, 1g, and 1i. When coupons were immersed in each sanitizer for 30 s and then steam treated for 20 s, most cells disappeared and those remaining were damaged as shown in Fig. 1d, 1f, 1h, and 1j.

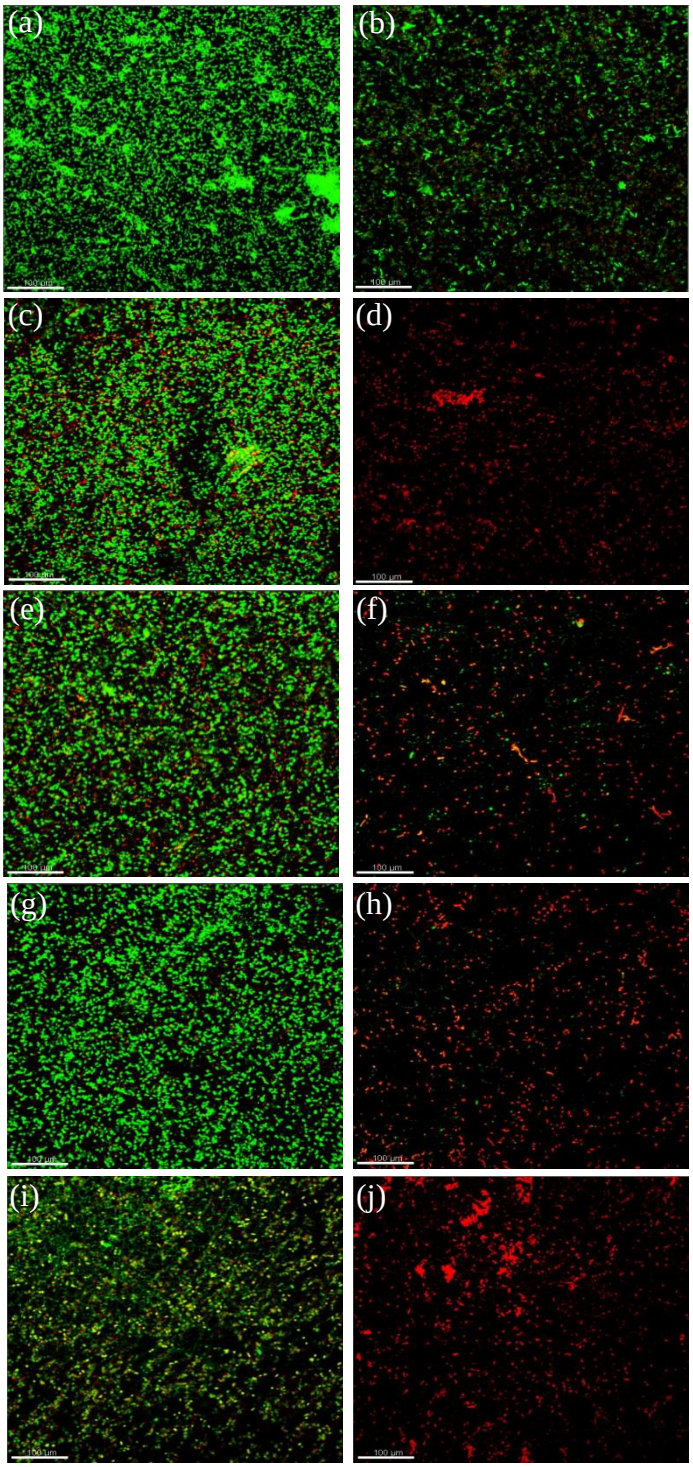

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)

Fig. I(1)-1. Membrane integrity of *E. coli* O157:H7 biofilm on stainless steel coupons observed by CLSM. The biofilm cells was treated with (a) D.W. (control), (b) steam for 20 s, (c) 100 ppm chlorine for 30 s, (d) 100 ppm chlorine for 30 s + steam for 20 s, (e) 2% hydrogen peroxide for 30 s, (f) 1% hydrogen peroxide for 30 s + steam for 20 s, (g) 100 ppm benzalkonium chloride for 30 s, (h) 100 ppm benzalkonium chloride for 30 s + steam for 20 s, (i) 100 ppm iodophor for 30 s, and (j) 100 ppm iodophor for 30 s + steam for 20 s (Green: viable; red: dead).

I(1)-4. Discussion

This study investigated the effectiveness of individual and combination treatments of steam and sanitizers for reducing foodborne pathogenic biofilm cells on the surface of stainless steel. Several disinfection methods using sanitizers have shown that bacteria in biofilms were more resistant than planktonic cells to the action of sanitizers. Lee Wong (1998) reported that biofilm populations on stainless steel were reduced by 3 to 5 log CFU/cm^2, while planktonic cells were reduced by 7 to 8 log CFU/ml following exposure to sanitizers. Also, Joseph et al. (2001) demonstrated that biofilm cells of *Salmonella* were more resistant to hypochlorite than were planktonic cells and could not be detected only when sanitizer treatment time exceeded 15 min. In the present study, individual treatments with, sanitizers were also insufficient to inactivate the three pathogens' biofilm cells on stainless steel.

Moreover, the effectiveness of each sanitizer was different for each of the three pathogens' biofilms. Inactivation of biofilm cells of foodborne pathogens using sanitizers has been investigated by several researchers. Joseph et al. (2001) observed that iodophor was more effective for inactivating biofilm cells of *Salmonella* compared to chlorine. Robbins et al. (2005) reported that biofilm cells of *L. monocytogenes* were reduced by 5.79 log CFU/chip following exposure to 100 ppm chlorine for 5 min and resulted in a 4.14 log CFU/chip reduction after 5% H_2O_2 exposure for 10 min. Sinde and Carballo (2000) reported that QAC was more effective against *Salmonella*

attached than for *L. monocytogenes* attached. In this study, BKC treatment alone was less effective at inactivating *L. monocytogenes* biofilm on stainless steel compared to other sanitizers. BKC was found to be ineffective against biofilm cells that existed below the first layer (Frank and Koffi, 1990). Because BKC are hydrophilic cationic molecules, they are able to penetrate hydrophilic and negatively charged cell surfaces (Bower, McGuire et al., 1996). However, lipophilic surfaces in the cell walls of gram-positive bacteria would most likely impede the penetration of sanitizers (Frank and Koffi, 1990).

In order to obtain complete inactivation of biofilm cells, long treatment times and high concentrations of sanitizers are needed. However, sanitizer treatment alone may not be adequate for use by food industry facilities because of the low sanitation effect shown in this study. Hence, sanitizer treatment needs to be developed as a practical and effective short-time food processing intervention for inactivating foodborne pathogen biofilm cells in combination with other control methods. Oh and Marshall (1995) reported that biofilm cells of *L. monocytogenes* were destroyed by 50 μg/ml monolaurin combined with heating at 65℃ for 5 min. Berrang et al. (2008) observed that ultrasound treatment did improve the effectiveness of quaternary ammonium and chlorine-based sanitizers. However, these combination methods are limited to difficult areas and have not yet found their way to practical application.

Steam treatment has proven to be one of the most effective methods for inactivating bacterial pathogens by virtue of its great heat capacity and ability to penetrate cavities and crevices (Trivedi et al., 2008). During steam treatment, the condensation of steam onto coupon surfaces produces a transfer of heat energy (latent heat), which causes rapid heating of the coupon surface, effectively destroying any pathogen biofilms (Castell-Perez and Moreira, 2004). Phebus et al. (1997) reported that steam treatment at 99–101℃ for 15 s reduced *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on surfaces of freshly slaughtered beef by 3.53, 3.74, and 3.44 log CFU/cm^2, respectively. Park and Kang (2014) observed that biofilm cells of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* were reduced to below the detection limit (1.48 log CFU/coupon) after steam treatment at 75℃ for 30–40 s, and at 85℃ for 20–30 s.

My previous study showed that the combination of steam treatment and lactic acid resulted in an additional log reduction as a result of their synergism compared to the sum of the reductions obtained after individual treatment of bacterial biofilms (Ban et al., 2012). We therefore investigated the combination effect of sanitizer and steam for inactivating bacterial biofilms.

In this study, when each sanitizer or steam treatment was applied to foodborne pathogen biofilm cells on stainless steel, small reductions in cell populations were observed. Combinations of each sanitizer and steam treatment resulted in greater reductions in three pathogens species' biofilm cell survival than did either treatment alone. These combinations achieved an

additional 0.01–2.78 log reduction of the three pathogens' biofilm cell levels compared to sum of the individual treatments (the synergistic effect). The most effective combination for reducing levels of pathogen biofilm cells was the combined treatment of steam and iodophor; steam for 20 s and merely 20 ppm iodophor for 30 s, which reduced cell numbers to below the detection limit (< 1.48 log CFU/coupon).

To date, no research has been published dealing with the effectiveness of sanitizer and steam combination treatments against pathogenic bacteria in biofilms. Accordingly, the results of this study can only be compared with results obtained in my previous study, which dealt with combinations of steam and lactic acid against the same pathogens (*E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*) (Ban et al. 2012). The synergistic effect of combinations of sanitizers and steam was similar to that of combinations of lactic acid and steam. In the current study, however, combinations of steam treatment and lower concentrations (20, 50, 100 ppm) of chemical agents (sanitizer: SHC, iodophor, and BKC, but not hydrogen peroxide) were required than for previously studied pathogen biofilms to reduce cell populations to below the detection limit (< 1.48 log CFU/coupon).

The performance of individual sanitizer depends on the target pathogen. Overall, Gram-negative bacteria were relatively more sensitive to sanitizer treatments than were Gram-positive bacteria in the present study. However, results indicate that combinations of steam and sanitizers were more effective at reducing foodborne pathogen biofilm cells to below the detection limit

compared to individual treatments, regardless of each sanitizer's actions and type of bacteria. Due to the high temperature and progressive steam treatment time, combined sanitizer and steam treatment may simultaneously provide cost-effectiveness and high-throughput processing on a large-scale for the food industry.

In conclusion, these overall results demonstrate that the combination treatment of sanitizers with steam produces a lethal effect by enhancing levels of inactivation of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* biofilm cells. Moreover, it is possible to decrease both sanitizer concentration and treatment time if used in combination with steam treatment. Therefore, combined sanitizer and steam treatment is a very promising alternative technology to control foodborne pathogen biofilm cells in food processing facilities as well as protecting foods from cross-contamination. The information obtained from this study will be helpful when developing strategies to inactivate foodborne pathogen biofilm cells on abiotic surfaces in food processing facilities by means of these base data. Also, combined effects between other techniques on foodborne pathogen biofilm cells which attach to abiotic surfaces such as glass, plastic, and wood should be researched. Furthermore, food residues such as fat and protein may still remain on the surface even if the biofilms cells are inactivated (Neu, 1992). These residues may lead to rapidly recolonize surface and result in equipment fouling. Therefore, organic residue removal step has to be preceded before application of this sanitation steps can occur.

Chapter I-(2)

Synergistic Effect of Steam and Lactic Acid against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* Biofilms on Polyvinyl Chloride and Stainless Steel

I(2)-1. Introduction

Foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* are a serious concern in food processing facilities. It is possible to occasionally find these pathogens in food processing facilities (Johansson et al., 1999; Hood and Zottola, 1997; Todd et al., 2009). *E. coli* O157:H7 is capable of causing bloody diarrhea and renal failure in humans (Doyle, 1991). *S.* Typhimurium causes diarrhea, fever, and abdominal cramps 12 to 72 hours after infection (Blaser and Newman, 1982). Listeriosis caused by *L. monocytogenes* results in meningitis, sepsis, encephalitis, febrile gastroenteritis, and abortion (Schlech, 2000).

Bacteria surrounded by extracellular polymeric substances can adhere to surfaces of food processing facilities, (Wingender, 1999), and aggregate in a hydrated polymeric matrix of their own synthesis to form biofilms (Costerton et al., 1999). Also, foodborne pathogens, e.g., *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes,* are able to form biofilms on abiotic surfaces and have enhanced tolerance to antibiotics or sanitizers when cells are in biofilms (Bower and Daeschel, 1999). Biofilms of foodborne pathogens not only present a considerable hygiene risk in the food industry due to providing a reservoir of contamination (Carpentier and Cerf, 1993), but also cause economic losses by causing technical failure in water systems, cooling towers, heat exchangers, and chain lubrication systems. (Meyer, 2003)

To kill and remove biofilm organisms from food processing facilities, various physical cleaning methods such as electric fields (Blenkinsopp et al., 1992), ultrasound (Mott et al., 1998), high pressure cleaning (Gibson et al., 1999; Meyer, 2003), and automatic scrubbers (Gibson et al., 1999; Meyer, 2003) have been evaluated. Also, inactivation of biofilm organisms with various chemical methods including alkali/acid wash (Flint et al., 1999; Parkar et al., 2004), chlorine (Meyer, 2003), peracid sanitizer (Fatemi and Frank, 1999), and acidic electrolyzed water (Ayebah et al., 2006) have been evaluated for their ability to inactivate biofilms on food processing facility surfaces. However, these methods are limited to small areas and have not yet found their way to practical application.

Steam treatment has been proven to be one of the most effective methods for inactivating bacterial pathogens and has been commercially validated for reducing microbial populations on beef carcasses (Trivedi et al., 2008). The main advantage of using steam is the large amount of heat transferred to food when steam condenses, which rapidly increases surface temperature (James et al., 2000). Steam at 100˚C has a greater heat capacity than the same amount of water at that temperature (James and James, 1997) and can effectively penetrate cavities, crevices and feather follicles that may provide protection for surface-attached microorganisms (Morgan et al., 1996). However, no studies have been conducted which have investigated the combination of steam and organic acid treatment for reducing biofilms.

In hurdle technology, combination treatments are utilized because it is expected that the use of combined factors will have greater effectiveness at inactivating microorganisms than the use of any single factor alone (Leistner, 2000). Lactic acid (LA) has been used in combination with steam in hurdle technology. Thermal destruction of bacteria using condensing steam is the most promising of the physical methods. The application of LA offers the best inactivation potential among various chemical methods (James et al., 1998). Incorporating LA with steam treatment is being evaluated for possible synergistic effect.

Therefore, the objective of this study was to determine and compare the effectiveness of individual treatments (steam and LA) and the combination of steam and LA for reducing foodborne pathogenic biofilm cells on PVC and stainless steel.

I(2)-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three isolates each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection of Seoul National University (Seoul, Korea) for this study. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37˚C for 24 h. Strains of each pathogen species were combined and cells were collected by centrifugation at 5000 g at 4℃ for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final pellets comprising separate pathogen species were resuspended in sterile PBS, corresponding to approximately 10^7–10^8 CFU/ml.

2.2. Preparation of PVC and stainless steel coupons

Schedule 80 PVC and stainless steel 316 (bright annealed) were cut into coupons (5 cm × 2 cm), immersed in 70% ethanol for 10 min to disinfect the surface, and rinsed with sterile distilled water. The washed PVC and stainless steel coupons were dried in a laminar flow biosafety hood (22 ± 2℃) for 3 h and sterilized by autoclaving before use.

2.3. Biofilm formation

Each prepared PVC and stainless steel coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing 30 ml of each cell suspension of *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* in PBS (ca. 10^7–10^8 CFU/ml). Conical centrifuge tubes containing coupons were incubated at 4℃ for 24 h to facilitate the attachment of cells. After incubation, coupons were removed with sterile forceps, immersed in 300 ml of sterile distilled water (22 ± 2℃) and gently stirred for 5 s. Washed coupons were transferred to 50 ml conical centrifuge tubes containing 30 ml of TSB (Tryptic soy broth), and maintained at 25℃ for 6 days of static incubation. The method used in this study was adapted from Kim et al. (2006).

2.4. Preparation of acid

LA (85.0–92.0%; Kanto Chemical Co. Inc., Tokyo, Japan) was used in this experiment. Treatment solutions of LA (0.5% and 2%, v/v) were prepared (22 ± 2℃) using sterile distilled water. The pH for 0.5% and 2% LA was 2.26 and 2.12, respectively.

2.5. Combination treatment of steam and acid

Coupons were removed and rinsed as described previously then immersed in LA for 5, 15, and 30 s, respectively. Then they were exposed to steam on

both sides for 5, 10, and 20 s, respectively, while maintaining an absolute pressure of 143 kPa. During these experiments, the distance between the coupons and the steam outlet was set at 40 mm. The coupons treated with steam alone and ones immersed into LA alone were used as controls.

2.6. Bacterial enumeration

After treatment, PVC and stainless steel coupons were placed in sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Immediately after vortexing, cell suspensions were tenfold serially diluted in buffered peptone water (BPW; Difco) , and 0.1 ml of undiluted cell suspension or diluents was spread-plated onto the appropriate selective medium (*E. coli* O157:H7: Sorbitol MacConkey Agar (SMAC), Difco; *S.* Typhimurium: Xylose Lysine Desoxycholate Agar (XLD), Difco; and *L. monocytogenes*: Oxford Agar Base with antimicrobic supplement BactoTM (MOX), Difco) to determine the number of biofilm cells attached to the surfaces of PVC and stainless steel coupons. Sorbitol MacConkey agar (SMAC; Difco), Xylose Lysine Desoxycholate agar (XLD; Difco), and Oxford agar base (OAB; Difco) with antimicrobic supplement (Difco) were used as selective media for the enumeration of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension was plated onto four plates of each respective

medium. The plates were incubated at 37℃ for 24–48 h. After incubation, colonies were counted.

2.7. Temperature monitoring

PVC and stainless steel coupon temperatures were monitored during steam treatment using a K-type Teflon-coated thermocouple. Probes were attached to the PVC and stainless steel coupons. The surface temperature was recorded every 1 s as the coupons were treated with steam.

2.8. Confocal laser scanning microscopy

A BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was used to examine bacterial cell membrane integrity. This kit includes SYTO9 and propidium iodide (PI) to distinguish between viable and damaged cell membranes, respectively. Viable cells were stained a green color, whereas damaged cells were stained red. After treatment with LA and steam, 0.1 mL of the staining solution was applied to *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* coated polyvinyl chloride (PVC) and stainless steel coupons and dried for 30 min in the dark. These samples were imaged with an upright confocal laser scanning microscope (CLSM, Eclipse 90i, Nikon, Japan) using a 60X water immersion objective lens with a numerical aperture of 0.9. Image stacks at various foci collected through the CLSM were reconstructed in three-dimension using IMARIS software (Bitplane, Zurich, Switzerland) (Park et al., 2010).

2.9. Statistical analysis

All experiments were repeated three times. Data was analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple range tests at a probability level of $P < 0.05$. All counts were normalized to 1 cm^2 areas and transformed into log values.

I(2)-3. Results

This study was performed to compare the effectiveness of steam treatment alone, organic acid treatment alone, and their combination on reducing the number of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* cells on PVC and stainless steel. In preliminary studies, we evaluated the antimicrobial effect of organic acids (propionic acid, acetic acid, lactic acid, malic acid, citric acid) on reducing foodborne pathogens (*E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*). Lactic acid was found to be the most effective among organic acids tested (data not shown). Therefore, lactic acid was chosen for this study.

3.1. Inactivation of E. coli O157:H7 *biofilm on PVC and stainless steel*

Table 1 shows survival of *E. coli* O157:H7 biofilm cells on PVC and stainless steel after steam and LA treatment. The initial level of *E. coli* O157:H7 biofilm on PVC and stainless steel was 6.21 and 6.17 log CFU/coupon, respectively. Slight reductions (< 0.5 log) occurred when inoculated samples were treated with water. This phenomenon was mainly due to physical removal of pathogen cells from PVC and stainless steel coupons.

The numbers of *E. coli* O157:H7 biofilm cells formed were significantly ($P < 0.05$) reduced as the concentration of LA and duration of steam treatment

increased.

PVC coupons immersed in LA alone experienced a log reduction range of 0.11–0.88 for *E. coli* O157:H7 biofilm cells, compared to that of the deionized water control. The reduction levels of *E. coli* O157:H7 biofilm cells were 0.34–1.75 log after steam treatment alone, but were 0.76–3.78 log after the combination treatment of steam and LA. It was observed that the combination treatment achieved an additional 0.31–1.15 log reduction compared to the sum of the individual treatments, indicating a synergistic effect. The levels of surviving biofilm cells on PVC coupons were reduced to below the detection limit (1.48 log CFU/coupon) when immersed in 2% LA for 15 or 30 s and then steamed for 10 s, or when immersed in 2% LA for 5, 15, or 30 s and then steam treated for 20 s.

The results showed a similar tendency on stainless steel coupons. However, the populations of *E. coli* O157:H7 biofilm cells on stainless steel were further reduced compared to biofilm on PVC and achieved an additional 0.52–2.11 log reduction.

Table I(2)-1. Survival (log CFU/coupon) of *Escherichia coli* O157:H7 in biofilm formed on the surface of PVC and stainless steel coupons treated with lactic acid and steam

		PVC			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	5.91±0.06 A a	5.57± 0.32 Aa	4.77±0.18 Ab	4.16±0.06 Ac
0.5	5	5.80±0.03 ABa	5.15±0.29 ABb	4.46±0.27 ABc	3.37±0.18 Bd
	15	5.74±0.01 ABa	4.94±0.51 ABb	4.08±0.17 ABc	3.11±0.05 BCd
	30	5.52±0.18 BCa	4.58±0.56 Bb	3.54±0.55 Bc	2.56±0.46 Dd
2	5	5.37±0.19 CDa	3.40±0.45 Cb	2.59±0.63 Cb	< 1.48 Dc
	15	5.22±0.27 CDa	2.42±0.18 Db	< 1.48 Dc	< 1.48 Dc
	30	5.03±0.33 Da	2.13±0.08 Db	< 1.48 Dc	< 1.48 Dc
		Stainless steel			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	6.12±0.16 A a	5.60±0.12 Ab	4.81±0.04 Ab	3.96±0.07 Ac
0.5	5	5.82±0.15 ABa	4.82±0.22 Bb	4.45±0.38 Ac	3.26±0.15 Bd
	15	5.61±0.20 BCa	4.50±0.16 BCb	3.86±0.19 Bc	2.87±0.33 Cd
	30	5.41±0.28 CDa	4.18±0.24 CDb	3.33±0.14 BCc	< 1.48 Dd
2	5	5.63±0.16 BCa	4.41±0.15 Cb	3.33±0.34 BCc	2.69±0.38 Cd
	15	5.43±0.17 CDa	3.82±0.24 DEFb	3.25±0.08 BCc	2.59±0.28 Cd
	30	5.12±0.15 DEa	3.59±0.38 FGb	2.50±0.49 Dc	< 1.48 Dd

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.2. Inactivation of S. Typhimurium *biofilms on PVC and stainless steel*

Table 2 shows survival of biofilm cells of *S.* Typhimurium after steam and LA treatment. The initial levels of *S.* Typhimurium biofilm cells on PVC and stainless steel were 6.19 and 6.32 log CFU/coupon, respectively.

S. Typhimurium biofilm cell populations on PVC coupons experienced a log range reduction of 0.13–1.08 in LA alone, compared to the deionized water control. The reduction levels of *S.* Typhimurium biofilm cells were 0.92–2.12 log after steam alone and 1.18–3.75 log after immersion in the combination treatment of steam and LA. The combination treatment resulted in an additional 0.13–2.01 log reduction compared to the sum of the individual treatments. The levels of surviving biofilm cells on PVC were reduced to below the detection limit (1.48 log CFU/coupon) when immersed in 2% LA for 15 or 30 s and then steamed for 10 s or by submersion in 2% LA for 5, 15, or 30 s and then steam treated for 20 s.

The results on stainless steel coupons showed a similar tendency. However, the populations of *S.* Typhimurium biofilm cells on stainless steel were further reduced compared to biofilm on PVC and resulted in an additional 0.24–1.58 log reduction.

Table I(2)-2. Survival (log CFU/coupon) of *Salmonella* Typhimurium in biofilm formed on the surface of PVC and stainless steel coupons treated with lactic acid and steam

		PVC			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	6.10±0.06 Aa	5.18±0.26 Ab	4.71±0.58 Ab	3.98±0.09 Ac
0.5	5	5.97±0.13ABa	4.92±0.16 Ab	4.56±0.52 Ab	3.45±0.76 Ac
	15	5.66±0.06 Ba	4.44±0.32 Bb	3.13±0.60 Bc	2.29±0.28 Bd
	30	5.29±0.12 Ca	3.59±0.18 Db	2.48±0.37 Bc	1.75±0.18 Bd
2	5	5.75±0.30 Ba	3.99±0.08 Cb	2.35±0.17 Bc	< 1.48 Cd
	15	5.33±0.24 Ca	3.15±0.04 Eb	< 1.48 Cc	< 1.48 Cc
	30	5.02±0.21 Ca	2.35±0.11 Fb	< 1.48 Cc	< 1.48 Cc
		Stainless steel			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	6.17±0.12 Aa	5.15±0.18 Ab	4.55±0.39 Ac	3.65±0.39 Ad
0.5	5	5.43±0.28 Ba	4.45±0.23 Ba	3.37±0.77 Bb	2.31±0.74 Bc
	15	5.32±0.32 Ba	4.06±0.49 Bb	2.46±0.72 Cc	1.70±0.21 BCc
	30	5.00±0.31 BCa	3.36±0.25 Cb	1.83±0.26 Cc	< 1.48 Dc
2	5	5.21±0.23 Ba	4.03±0.22 Bb	2.01±0.24 Cc	< 1.48 Dd
	15	4.85±0.48 BCa	2.88±0.41 CDb	< 1.48 Dc	< 1.48 Dc
	30	4.56±0.45 Ca	2.30±0.47 Db	< 1.48 Dc	< 1.48 Dc

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.3. Inactivation of L. monocytogenes biofilms on PVC and stainless steel

Table 3 shows surviving biofilm cells of *L. monocytogenes* enumerated on OAB agar after treatment. The initial levels of *L. monocytogenes* biofilm cells were 6.42 and 6.38 log CFU/coupon for PVC and stainless steel, respectively. The numbers of *L. monocytogenes* biofilm cells on PVC coupons were reduced by 0.19–0.94 log in LA alone. Cell numbers were reduced by 1.14–1.92 log in steam alone and 1.42–3.99 log in LA following the combined treatment of steam and LA. The combination treatment achieved an additional 0.28–1.89 log reduction compared to the sum of the individual treatments. The levels of surviving biofilm cells on PVC were reduced to below the detection limit (1.48 log CFU/coupon) when submerged in 2% LA for 30 s and then steam treated for 10 s or when immersed in 2% LA for 15 or 30 s and then steamed for 20 s, respectively.

The results for stainless steel coupons showed a similar tendency. However, populations of *L. monocytogenes* biofilm cells on stainless steel were further reduced compared to biofilm on PVC and resulted in an additional 0.24–1.77 log reduction.

Table I(2)-3. Survival (log CFU/coupon) of *Listeria monocytogenes* in biofilm formed on the surface of PVC and stainless steel coupons treated with lactic acid and steam

		PVC			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	6.24±0.14 A a	5.10±0.13 Ab	4.69±0.22 Abc	4.32±0.36 Ac
0.5	5	6.05±0.07 ABa	4.82±0.12 Ab	4.13±0.12 ABc	3.76±0.29 ABd
	15	5.87±0.09 ABCa	4.18±0.46 Bb	3.74±0.31 BCbc	3.06±0.64 BCc
	30	5.56±0.40 CDa	3.27±0.15 Cb	3.03±0.14 Cb	2.25±0.05 Cc
2	5	5.87±0.16 BCa	3.85±0.26 Bb	3.22±0.23 Cc	2.36±0.41 Cd
	15	5.75±0.09 BCa	3.28±0.22 Cb	2.31±0.17 Dc	< 1.48 Dd
	30	5.30±0.39 Da	2.49±0.27 Db	< 1.48 Ec	< 1.48 Dc
		Stainless steel			
		Treatment time (s)			
Acid (%)	Immer-sion (s)	0	5	10	20
0	-	6.09±0.11 Aa	4.83±0.19 Ab	4.58±0.18 Ab	3.91±0.06 Ac
0.5	5	5.94±0.10 ABa	4.44±0.26 ABb	4.02±0.19 Abc	3.56±0.37 ABc
	15	5.73±0.18 ABCa	4.26±0.13 ABb	3.71±0.50 ABbc	3.14±0.53 Bc
	30	5.50±0.35 BDCa	3.57±0.24 Cb	2.83±0.13 BCc	2.15±0.05 Cd
2	5	5.45±0.25 CDa	3.94±0.49 BCb	3.06±0.66 Bc	< 1.48 Dd
	15	5.31±0.33 CDa	3.23±0.48 Cb	2.03±0.45 Cc	< 1.48 Dd
	30	5.17±0.33 Da	2.22±0.56 Db	< 1.48 Dc	< 1.48 Dc

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.4. Temperature monitoring

The mean surface temperatures of PVC and stainless steel coupons during steam treatment are shown in Fig. 1. The surface temperatures of PVC reached the 75℃ treatment temperature within 14 to 15 s. The stainless steel coupon temperature rose quickly, and reached treatment temperature within approximately 5 s.

3.5. Effect of hyperthermia on membrane integrity

Disintegration of the bacterial cell membrane in *E. coli* O157:H7 biofilm following LA and steam treatment was observed through confocal laser scanning microscopy. Fig. 2 shows that destruction of the cell membrane in the *E. coli* O157:H7 biofilm was dependent on the concentration of LA and steam duration. The CLSM image of the *E. coli* O157:H7 biofilm was captured after treatment with the water control, immersion in 2% LA for 30 s, exposure to steam for 20 s, and the combination of LA and steam. The green and red stained cells indicate those having intact and damaged cell membranes (i.e., live and damaged/dead cells), respectively. When the *E. coli* O157:H7 biofilm was treated with distilled water without steam and LA exposure, almost all of the cells remained intact, as shown in Fig. 2a. Fig. 2c shows that the range of red-stained cells increased after steam treatment for 20 s. When coupons were immersed in 2% LA for 30 s and then steam treated for 20 s, most cells disappeared and those remaining were damaged.

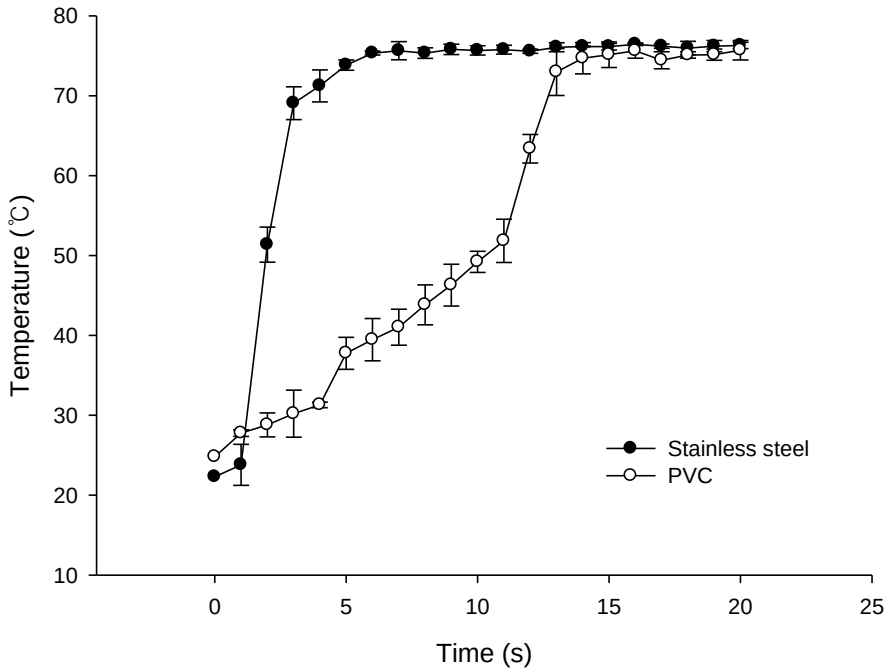


Fig. I(2)-1. Temperature changes versus treatment time on PVC and stainless steel coupons.

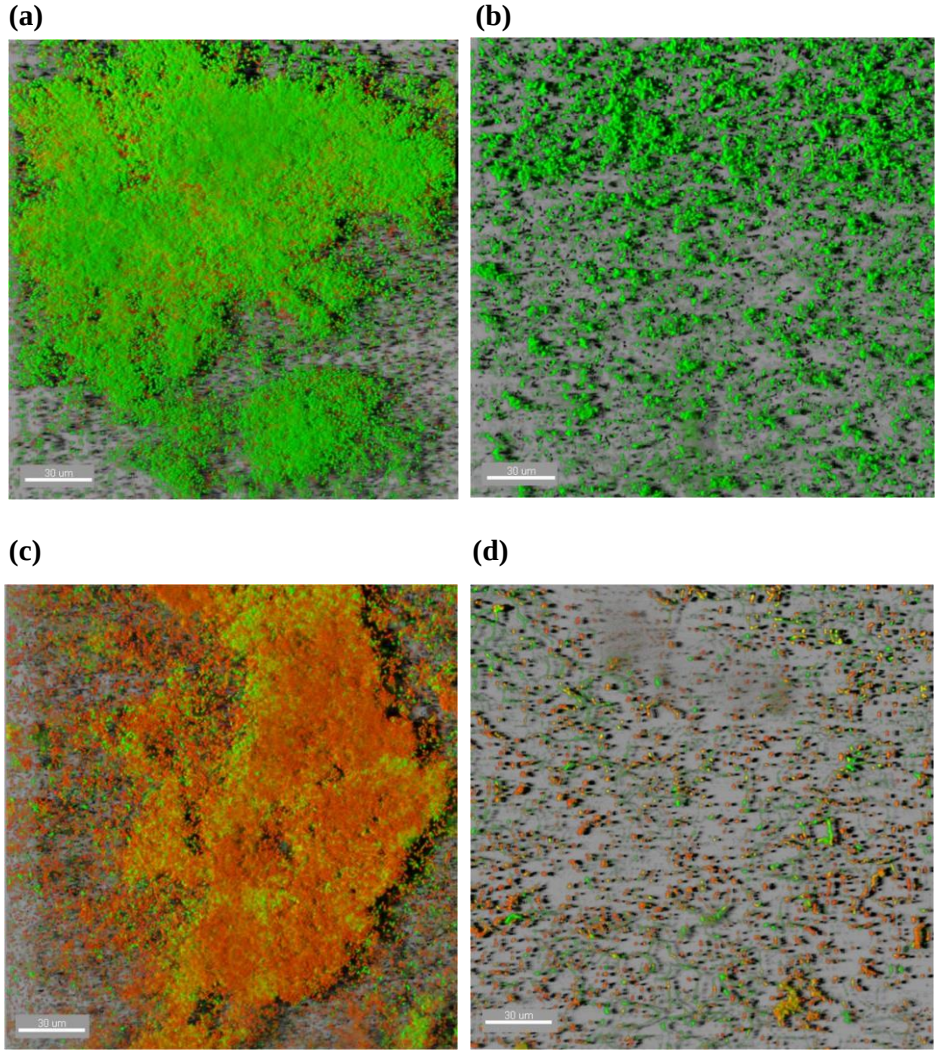


Fig. I(2)-2. Membrane integrity of *E. coli* O157:H7 biofilm on stainless steel observed by CLSM. The biofilm was treated with (a) D.W., (b) 2% lactic acid for 30 s, (c) steam for 20 s, and (d) 2% lactic acid for 30 s + steam for 20 s (Green: viable; red: dead).

I(2)-4. Discussion

When steam alone was applied to foodborne pathogen biofilm cells on PVC and stainless steel, there were significant biofilm cell reductions at all treatment times (5, 10, and 20 s) ($P < 0.05$). Reduction of pathogen biofilms by steam treatment is mainly due to latent heat (Huang, 2004). During steam treatment, the condensation of steam onto coupon surfaces produces a transfer of heat energy (latent heat), which causes rapid heating of the coupon surface, effectively destroying any pathogen biofilms (Castell-Perez and Moreira, 2004). Coupon temperature results may explain why the steam treatment was somewhat less effective on PVC coupons. Based on the temperature graphs in Fig. 1, bacterial populations on PVC coupons did not receive the same thermal effect as stainless steel coupons. When heat energy transfers to coupon surfaces, the thermal conductivity of stainless steel (16 W/m·K) is higher than that of PVC (0.19 W/m·K). This supports the results of this study. The kind of coupon significantly influences the reduction of the three pathogens' biofilm cells. After steam treatment, the three pathogens' biofilm cells experienced further reduction on stainless steel compared to PVC coupons. The maximum reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on PVC were 1.75, 2.12, and 1.92 CFU/coupon and on stainless steel were 2.27, 2.52, and 2.18 log CFU/coupon, respectively, after treatment with steam alone for 20 s. These results indicate that treatment with low pressurized steam alone may not be adequate for use by the food industry

because of the low sanitation effect. Even though high pressurized steam is particularly effective in decontamination, capital costs of high-pressure steam equipment increase exponentially with operating pressures. It is therefore economically beneficial to use low pressure steam in combination with other methods to inactivate foodborne pathogens. (Chen, 2007) Hence, steam treatment needs to be developed as a practical and effective short-time food processing intervention for inactivating and detaching foodborne pathogens in combination with other control methods such as aqueous sanitizers, hot water, and ultrasound (Jensen, 1998; Chmielewski and Frank, 2004; Morild, 2011).

As an individual treatment, lactic acid also produced significant reductions of the three pathogens' biofilms on PVC and stainless steel ($P < 0.05$). The mechanism of inhibition of microorganisms by organic acids, such as lactic acid, is related to several factors including reduction in pH, the ratio of the undissociated form of the acid, chain length, degree of branching, cell physiology and metabolism (Doores, 1995). It is known that weak organic acids are more inhibitory than strong acids because they are lipophilic and penetrate the plasma membrane and thus acidify the cell's interior (Ita, 1991). Reduction levels of the three pathogens increased as pH decreased from 2.26 to 2.12 in the solution depending on the concentration of lactic acid (0.5 to 2.0%). Reduction levels of pathogens following treatment with lactic acid increased with increasing lactic acid concentration.

In this study, the combined treatment of steam and lactic acid was employed to inhibit three pathogens' biofilm cells on PVC and stainless steel.

This combination achieved an additional ≥ 0.66 log reduction of the three pathogens' biofilm cell levels compared to lactic acid treatment alone. The most effective combination for reducing levels of pathogen biofilm cells was the combined treatment of steam for 20 s and 2% lactic acid for 15 s or 30 s which reduced cell numbers to below the detection limit (1.48 log). Other researchers have studied the effects of steam with lactic acid for killing and detaching pathogens on chicken, cooked frankfurters and other foods. Lecompte et al. (2008) reported that the reduction of *L. innocua* on chicken skins was 4.56 log CFU/g observed after the combined treatment of steam at 98℃ for 10 s followed by 10% lactic acid for 30 min. This reduction was greater than for steam or lactic acid treatment alone. Murphy et al. (2006) reported that combined treatments of organic acid and steam were effective at killing *L. monocytogenes* on fully cooked frankfurters. The reduction of *L. monocytogenes* was 3.43 log CFU/cm^2 observed after the combined treatment of steam at 105℃ for 1.5 s and organic acid solution, and no growth of *L. monocytogenes* was detected after the frankfurters were stored at 7℃ for 14 weeks or at 4℃ for 19 weeks. This study results may be due to lactic acid penetration of the cell membrane combined with latent heat generated by steam resulting in enhanced inactivation of pathogen biofilms on PVC and stainless steel coupons. More of the acid would be undissociated at a lower rather than a neutral pH. Because of the loss of H1-ATPase when bacteria are exposed to low pH, sublethal injury may occur to the cells by disrupting the proton motive force across cell membranes (Eklund, 1983). Therefore, after

low pH exposure, bacterial biofilms may be more susceptible to heat or other antimicrobial chemicals.

This study results indicate that effectiveness of steam and lactic acid treatment depends on the target pathogen. *S.* Typhimurium was relatively more sensitive to heat and acid treatment than *E. coli* O157:H7 and *L. monocytogenes*. Similar results were observed in other investigations. Benjamin and Datta (1995) reported that *E. coli* O157:H7, but not all strains, is acid tolerant. Similarly, *L. monocytogenes* is relatively tolerant to heat and acid (Skandamis et al. 2008). Compared to *L. monocytogenes*, *E. coli* O157:H7 was found to be more sensitive to the lethal effect of acid and heat treatment. The resistance of *L. monocytogenes* may be related to some inherent property of the organism itself. In addition, this result may be attributed to the difference between Gram-negative and Gram-positive characteristics of the bacteria. Gram-negative bacteria are commonly more susceptible at low pH and heat treatment than Gram-positive bacteria (Ray and Sandine, 1992).

These results indicate that the combination of steam treatment and lactic acid has a lethal effect on bacterial biofilms and demonstrate that food processing facilities could increase the microbial safety of products while simultaneously decreasing acid concentration and shortening processing time if they used the combination of steam and organic acids. This study confirms that the combination of steam and lactic acid is an effective alternative for improving inactivation of biofilms of three pathogens on PVC and stainless

steel. Statistical analysis of the differences in the survivability of biofilm cells of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* between combination and non-combination treatments revealed a strong synergistic effect between steam and lactic acid ($P < 0.05$). It is suggested that the combination of lactic acid and steam has potential as a disinfectant for food processing facilities. For effective utilization by the food processing industry, all food processing facilities should be constructed to resist acid because of the corrosive nature of lactic acid. And it is suggested that a pre-treatment removal of proteins prior to application of steam and lactic acid is needed to control recolonization of the surface by pathogens. Even if the biofilms cells are inactivated, biofilm and food residues may still remain on the surface (Neu, 1992). These residues may enhance microbial attachment resulting in a rapidly recolonized surface. Therefore before commercial application of this method can occur, additional studies about food residues and recolonization will need to be investigated.

Chapter I(3)

A Comparison of Saturated Steam and Superheated Steam for Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* Biofilms on Polyvinyl Chloride and Stainless Steel

I(3)-1. Introduction

Bacteria can attach to solid surfaces of food processing facilities (Wingender et al., 1999) and form slimy, slippery biofilms consisting of hydrated extracellular polymeric substances (Costerton et al., 1999). Adhesion of bacteria to food processing facility surfaces leads to potential hygienic problems in the food processing industry because the resultant surface-adherent pathogenic biofilms transmit pathogens to food (Barnes et al., 1999; Shi and Zhu, 2009). Bacteria can form biofilms on stainless steel, polyvinyl chloride (PVC), glass, and rubber (Pedersen, 1990; Prouty and Gunn, 2003; Ronner and Wong, 1993) and exhibit increased resistance to cleaning and disinfection compared to planktonic cells (Bower and Daeschel, 1999; Mah and O'Toole, 2001).

Escherichia coli O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are typical foodborne pathogens capable of producing biofilms on food processing facility surfaces (Chae and Schraft, 2000; Prouty and Gunn, 2003; Ryu and Beuchat, 2005). *E. coli* O157:H7 is an important pathogen causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). The principal symptoms of *S.* Typhimurium infection are diarrhea, fever, and abdominal cramps occurring within 12 to 72 hours postinfection (Baird-Parker, 1990; Blaser and Newman, 1982). Listeriosis caused by *L. monocytogenes*, with a

mortality rate of about 24%, results in abortion, neonatal death, septicemia, and meningitis (Farber and Peterkin, 1991; Schlech and Acheson, 2000).

Foodborne pathogen biofilms are responsible, in part at least, for food processing plant technical failures in water systems, cooling towers, heat exchangers, and chain lubrication systems and threaten the quality of food products by forming a reservoir of contamination (Flint et al., 1999; Meyer, 2003). In order to inactivate and remove biofilm organisms from food processing facilities, various cleaning methods have been evaluated to inactivate biofilms, including high pressure (Gibson et al., 1999; Meyer, 2003), electric fields (Blenkinsopp et al., 1992), ultrasound (Mott et al., 1998), automatic scrubbers (Gibson et al., 1999; Meyer, 2003), catalyst modified surfaces (Wood et al., 1996), various chemical methods, e.g. alkali/acid wash (Flint et al., 1999; Parkar et al., 2004), and proteolytic enzyme-based cleaning (Flint et al., 1999). However, these methods are usually expensive and difficult to implement and are applicable only to small areas (Meyer, 2003).

Superheated steam (SHS) is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure (Cenkowski et al., 2007). SHS has been proven to be one of the most effective methods for the drying of biological or nonbiological products, including foods (Braud et al., 2001; Taechapairoj et al., 2006). However, the inactivation of bacteria or bacterial spores by SHS has rarely been studied (Spicher et al., 2002). SHS has various advantages over other heating systems, such as a high rate of heat transfer due to condensation and gas radiation,

accelerated drying rates, and an oxygen-free environment (Bari et al., 2010). The main advantage of using SHS is the large amount of heat transferred to food when steam condenses on food surfaces, which rapidly increases the surface temperature (James et al., 2000). SHS, like SS, can effectively penetrate cavities, crevices and feather follicles that may provide protection for surface-attached microorganisms while water cannot reach all the contaminated surfaces because of the high surface tension of aqueous fluids (Morgan et al., 1996). Also, SHS rapidly releases heat of vaporization through condensation compared to SS when it condenses on surfaces because SHS has a higher enthalpy than that of the SS (Keyes et al, 1936). However, no studies have been conducted to evaluate SHS treatment for reducing biofilms.

Therefore, the purpose of this study was to compare and evaluate the effectiveness of SS and SHS for inactivating three foodborne pathogenic biofilm cells on the surfaces of PVC and stainless steel.

I(3)-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80℃ in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 15% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37℃ for 24 h, and stored at 4℃. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37℃ for 24 h. Cells of each strain were collected by centrifugation at 5000 g at 4℃ for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final pellets were resuspended in sterile PBS, corresponding to approximately 10^7–10^8 colony-forming units (CFU)/ml.

2.2. Preparation of PVC and stainless steel coupons

Schedule 80 PVC and type 316 stainless steel coupons with no. 4 finish (5 cm × 2 cm) were used. These coupons were immersed in 70% ethanol for 60 min to disinfect the surface, and rinsed with sterile distilled water. The

washed PVC and stainless steel coupons were dried in a laminar flow biosafety hood (22 ± 2℃) for 3 h and sterilized by autoclaving before use.

2.3. Biofilm formation

Each prepared PVC and stainless steel coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing suspensions (30 ml) of either *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* in PBS (ca. 10^7–10^8 CFU/ml). Coupons in bacterial cell suspensions (10^7–10^8 CFU/ml) were incubated at 4℃ for 24 h to facilitate the attachment of cells and the coupons were then removed with sterile forceps, immersed in 300 ml of sterile distilled water (22 ± 2℃) and gently stirred for 5 s. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of TSB, and then incubated at 25℃ for 6 days. This method was adapted from Kim et al. (2006).

2.4. Saturated steam (SS) and superheated steam (SHS) treatment

Coupons were removed from the tubes and rinsed as described previously then exposed to SS and SHS on both sides for 5, 10, 20 and 30 s, respectively. During these experiments, the SS and SHS temperature was controlled automatically by means of a temperature sensor and intelligent power module in the steam generator. SS treatments were performed at 100℃ while SHS treatments were performed at temperatures of 125, 150, 175, and 200℃.

2.5. Bacterial enumeration

After SS and SHS treatment, PVC and stainless steel coupons were deposited in sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of sterile glass beads (425-600 μm; Sigma-Aldrich, St. Louis, MO, USA) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Cell suspensions in tubes were tenfold serially diluted in buffered peptone water (BPW; Difco) , and then 0.1 ml of undiluted cell suspension or diluents were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate the number of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* biofilm cells, respectively, attached to the surfaces of PVC and stainless steel coupons. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension were plated onto four plates of each respective medium. The plates were incubated at 37℃ for 24–48 h and colonies were counted.

2.6. Temperature monitoring

PVC and stainless steel coupon temperatures were monitored during SS and SHS treatment using a K-type Teflon-coated thermocouple. Probes were attached to PVC and stainless steel coupons. Surface temperatures were recorded every 2 s while coupons were treated with SS and SHS.

2.7. Confocal laser scanning microscopy

A BacLight Live/Dead bacterial viability kit (L-7012, Molecular Probes, USA) was applied to biofilm coupons in order to evaluate cell membrane integrity. The kit included SYTO9 and propidium iodide (PI) to distinguish between intact and damaged cell membranes, respectively. The stain was prepared by diluting 3 μl of each component into 1 ml of distilled water. After SS and SHS treatment, 0.1 mL of the staining solution was applied to the biofilm coupons and stained for 30 min at room temperature in the dark. Viable cells appeared green in color, whereas damaged cells were stained red. Biofilm samples were imaged with an upright confocal laser scanning microscope (CLSM, Eclipse 90i, Nikon, Japan) using a 60X water immersion objective lens with a numerical aperture of 0.9. Image stacks at various foci collected through the CLSM were reconstructed in three-dimension using IMARIS software (Bitplane, Zurich, Switzerland) (Park et al., 2010).

2.8. Statistical analysis

All experiments were repeated three times with duplicate samples. Data was analyzed by analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple range test at a probability level of $P < 0.05$.

I(3)-3. Results

3.1. Inactivation of E. coli O157:H7 *biofilm on PVC and stainless steel*

Table 1 shows survival of *E. coli* O157:H7 biofilm cells on PVC and stainless steel after SS and SHS steam treatment. The initial level of *E. coli* O157:H7 biofilm on PVC and stainless steel was 6.35 and 6.24 log CFU/coupon, respectively. Slight reductions (< 0.5 log) occurred when inoculated samples were rinsed in water for controls (data not shown). This phenomenon was mainly attributable to the physical removal of biofilm cells from PVC and stainless steel coupons during washing.

The levels of *E. coli* O157:H7 biofilm cells survived were significantly ($P < 0.05$) reduced as the duration of SHS treatment increased. *E. coli* O157:H7 biofilm cells experienced a log reduction range of 1.21–2.64 after SS treatment for 5–30 s, but were reduced by 1.26–4.87 log to below the detection limit (1.48 log CFU/coupon), after SHS treatment for the same time. It was observed that the SHS treatment caused an additional 0.05–2.23 log reduction compared to the SS treatments. The populations of viable biofilm cells on PVC coupons were reduced below the detection limit when subjected to SHS treatment for 20 s at 200℃ and for 30 s at 175 and 200℃.

The results for stainless steel coupons showed a similar tendency. However, biofilms on stainless steel showed more significant reduction in viable cell numbers when compared with PVC biofilms, leading to reduction

below the detection limit (1.48 log CFU/coupon) when heat-treated with SHS for 20 and 30 s at 150, 175 and 200℃ and for 10 s at 200℃.

Table I(3)-1. Survival (log CFU/coupon) of *Escherichia coli* O157:H7 in biofilm formed on the surface of PVC and stainless steel coupons treated with saturated steam and superheated steam

Steam temperature (℃)	PVC			
	Treatment time (s)			
	5	10	20	30
100	5.14±0.12 Aa	4.73±0.09 Ab	4.19±0.12 Ac	3.71±0.11 Ad
125	5.09±0.02 Aa	4.55±0.14 ABb	3.86±0.07 Bc	2.56±0.23 Bd
150	4.99±0.18 ABa	4.25±0.56 BCb	3.56±0.55 Cc	1.49±0.46 Cd
175	4.77±0.19 BCa	4.20±0.45 BCa	2.59±0.63 Db	< 1.48 Cc
200	4.68±0.27 Ca	4.06±0.18 Ca	< 1.48 Eb	< 1.48 Cb
Steam temperature (℃)	Stainless steel			
	Treatment time (s)			
	5	10	20	30
100	4.73±0.07 Aa	4.20±0.25 Aa	3.44±0.12 Ab	2.48±0.11 Ac
125	4.40±0.01 ABa	3.58±0.06 Bb	3.35±0.01 Ab	2.11±0.09 Ac
150	4.21±0.08 Ba	3.31±0.77 Bb	< 1.48 Bc	< 1.48 Bd
175	3.42±0.18 Ca	2.21±0.26 Cb	< 1.48 Cc	< 1.48 Bc
200	2.82±0.19 Da	< 1.48 Db	< 1.48 Cc	< 1.48 Bc

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.2. Inactivation of S. Typhimurium *biofilms on PVC and stainless steel*

Table 2 shows survival of biofilm cells of *S.* Typhimurium after SS and SHS treatment. Prior to steam exposure, the cell numbers on PVC and stainless steel biofilms were equivalent to 6.51 and 6.49 log CFU/coupon, respectively.

SS treatment for 5-30 s brought about 1.35 to 3.43 log decreases in *S.* Typhimurium biofilm cells, whereas SHS treatment reduced bacterial cell numbers below the detection limit, showing 1.42–5.03 log decrease for 5–30 s. An additional reduction of 0.07 to 2.60 log reduction was observed in SHS treatment, compared to SS treatments. In the case of PVC biofilms, viable bacterial cells were reduced below the detection limit by 20 s SHS treatment at 175 and 200℃ or 30 s SHS treatment at 150, 175 and 200℃.

Comparing steam inactivation efficiency between PVC and stainless steel, stainless steel biofilms exhibited more significant cellular reductions by steam treatment, showing reduction below the detection limit when subjected to SHS treatment for 10 s at 200℃, or for 20 and 30 s at 150, 175, and 200℃.

Table I(3)-2. Survival (log CFU/coupon) of *Salmonella* Typhimurium in biofilm formed on the surface of PVC and stainless steel coupons treated with saturated steam and superheated steam

Steam temperature (℃)	PVC			
	Treatment time (s)			
	5	10	20	30
100	5.16±0.13 Aa	4.36±0.16 Ab	3.61±0.52 Ac	3.08±0.76 Ad
125	5.09±0.06 Aa	4.25±0.32 ABb	2.99±0.60 ABc	2.29±0.28 Bd
150	5.07±0.06 Aa	4.14±0.32 ABa	2.79±0.60 Bb	< 1.48 Cc
175	4.95±0.30 Aa	3.99±0.08 Bb	< 1.48 Cc	< 1.48 Dc
200	4.88±0.24 Aa	3.28±0.04 Cb	< 1.48 Cc	< 1.48 Dc
Steam temperature (℃)	Stainless steel			
	Treatment time (s)			
	5	10	20	30
100	4.79±0.28 Aa	3.96±0.23 Ab	2.90±0.77 Abc	2.39±0.74 Ac
125	3.82±0.32 Aa	2.56±0.49 Bb	2.46±0.72 Ab	1.70±0.21 Bc
150	3.24±0.31 Ba	2.38±0.25 Bb	< 1.48 Bc	< 1.48 Cc
175	3.21±0.23 Ba	2.03±0.22 Cb	< 1.48 Bc	< 1.48 Cc
200	2.75±0.48 Ba	< 1.48 Db	< 1.48 Bb	< 1.48 Cb

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.3. Inactivation of L. monocytogenes biofilms on PVC and stainless steel

Table 3 shows surviving biofilm cells of *L. monocytogenes* after steam treatment enumerated on OAB agar. The cell numbers of *L. monocytogenes* biofilms were 6.32 and 6.17 log CFU per PVC and stainless steel coupons, respectively, before steam treatment.

SS treatment resulted in 1.34–2.52 log reductions in surviving biofilm cells, while SHS treatment caused 1.45–4.84 log reduction for the equivalent times. SHS treatment achieved an additional 0.11–2.32 log reduction in cell numbers compared to SS treatments. PVC biofilm cells were fully inactivated when steam-treated for 30 s at 175 and 200℃, showing reduction below the detection limit.

Stainless steel biofilms also showed a similar inactivation tendency under steam treatment. However, *L. monocytogenes* biofilm cells on stainless steel coupons were more damaged compared to PVC biofilm cells and were reduced below the detection limit when exposed to superheated steam for 10 s at 200℃, and 20 and 30 s at 150, 175, and 200℃.

Table I(3)-3. Survival (log CFU/coupon) of *Listeria monocytogenes* in biofilm formed on the surface of PVC and stainless steel coupons treated with saturated steam and superheated steam

Steam temperature (℃)	PVC			
	Treatment time (s)			
	5	10	20	30
100	4.98±0.07 Aa	4.38±0.12 Ab	4.15±0.12 Ab	3.80±0.29 Ac
125	4.87±0.09 Aa	4.18±0.46 ABb	3.74±0.31 Bbc	3.06±0.64 Bd
150	4.52±0.40 Ba	3.90±0.15 Bb	3.54±0.14 BCb	2.24±0.05 Cc
175	4.46±0.16 Ba	3.85±0.26 Bb	3.22±0.23 Cc	< 1.48 Dd
200	4.44±0.09 Ba	3.45±0.22 Cb	2.44±0.17 Dc	< 1.48 Dd
Steam temperature (℃)	Stainless steel			
	Treatment time (s)			
	5	10	20	30
100	4.79±0.10 Aa	4.47±0.26 Aab	3.74±0.19 Ab	3.16±0.37 Ac
125	3.73±0.18 Aa	3.26±0.13 Ab	2.71±0.50 Bc	2.14±0.53 Ad
150	3.56±0.35 Aa	2.53±0.24 Bb	< 1.48 Bc	< 1.48 Bc
175	2.45±0.25 Ba	1.94±0.49 Bc	< 1.48 Bc	< 1.48 Bc
200	2.22±0.33 Ba	< 1.48 Bb	< 1.48 Bb	< 1.48 Bb

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.4. Temperature monitoring

The mean surface temperatures of PVC and stainless steel coupons during SS or SHS treatment are shown in Fig. 1. The surface temperatures of PVC exposed to SS treatment reached 70℃ within 18 s while stainless steel coupon reached the same temperature within approximately 8 s. Stainless steel enabled faster temperature increase than PVC during steam treatment and the higher the applied temperature, the greater difference in the final temperature two types of coupons generated.

3.5. Effect of hyperthermia on membrane integrity

Disintegration of the bacterial cell membrane in *E. coli* O157:H7 biofilm following SS and SHS treatment was observed through confocal laser scanning microscopy. BacLight Live/Dead-stained biofilms were imaged and showed that destruction of the cell membrane in *E. coli* O157:H7 biofilm was dependent on the property of steam and heating duration (Fig. 2). CLSM images of *E. coli* O157:H7 biofilms were captured after treatment with the water control, exposure to SS at 100℃, and to SHS at 150 and 200℃ for 20 s. Live cells, which have intact membranes, were stained with SYTO9 and showed a green fluorescence, while damaged cells stained with PI and emitted a red fluorescence. Untreated *E. coli* O157:H7 biofilm was stained mostly green, with a few red cells being present, as shown in Fig. 2a. Fig. 2b, 2c and 2d show that the range of red-stained cells increased after SS and SHS treatment for 20 s as treatment temperature increased. When coupons were

treated with SHS for 20 s at 200℃, most biofilm cells disappeared and those remaining were damaged.

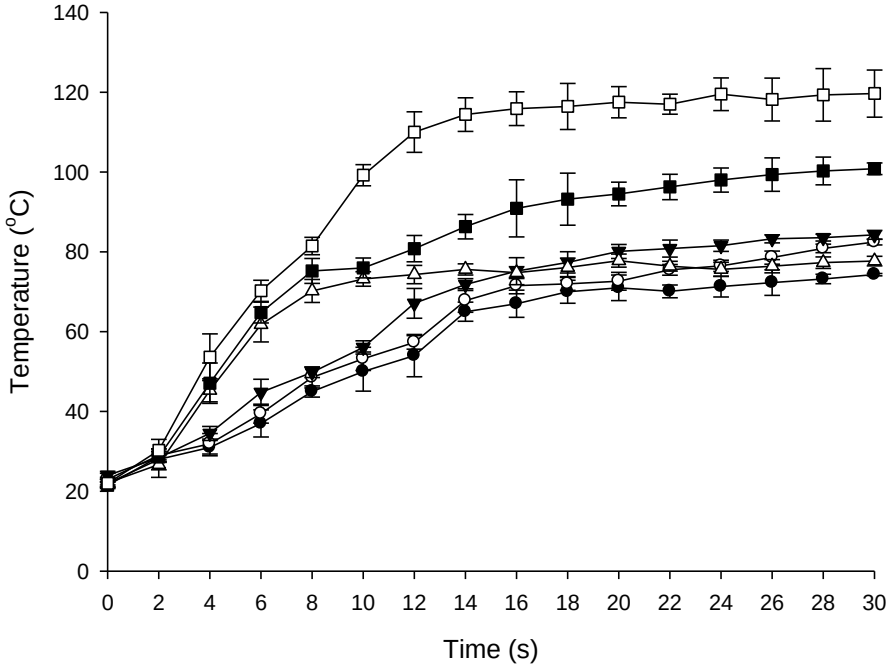


Fig. I(3)-1. Temperature changes versus treatment time on PVC and stainless steel coupons. ●, PVC treated with saturated steam at 100°C; ○, PVC treated with superheated steam at 150°C; ▼, PVC treated with SHS at 200°C; △, Stainless steel treated with SS at 100°C; ■, stainless steel treated with SHS at 150°C; □, stainless steel treated with SHS at 200℃.

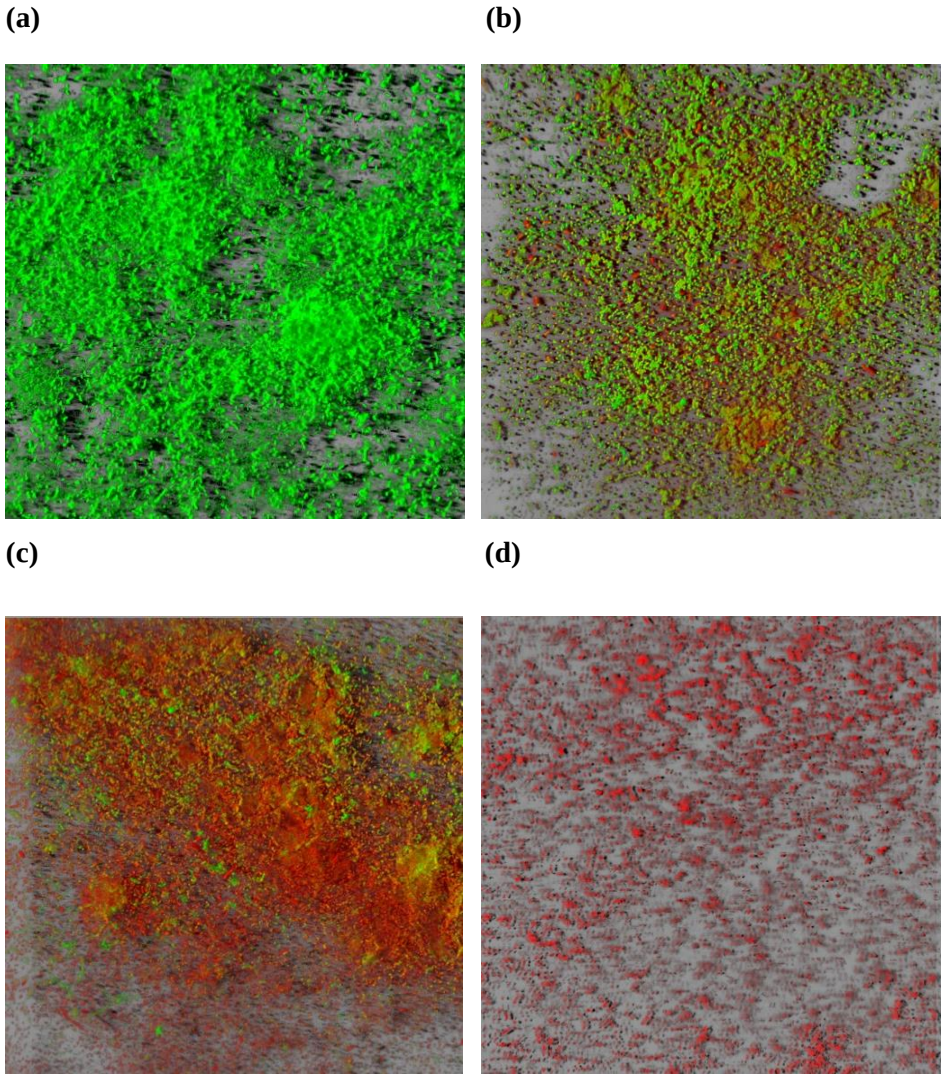


Fig I(3)-2. Membrane integrity of *E. coli* O157:H7 biofilm on stainless steel observed by CLSM. The biofilm was treated with (a) D.W., (b) saturated steam at 100℃ for 20 s, (c) superheated steam at 150℃ for 20 s, and (d) superheated steam at 200℃ for 20 s observed by CLSM (Green: viable; red: dead).

I(3)-4. Discussion

Foodborne pathogen biofilms are implicated in food industrial biofouling, microbial regrowth in the distribution system, continuous infections, and numerous other costly and life-threatening problems (Kim et al., 2008; Kumar and Anand, 1998; Møretrø and Langsrud, 2004). Consequently, the control of bacteria in biofilms is of extreme importance and SHS was proven to be an effective method for inactivating biofilms cells on PVC and stainless surfaces commonly used in food processing facilities.

When SS and SHS was applied to foodborne pathogen biofilm cells on PVC and stainless steel, significant reductions in biofilm cells were observed at all tested treatment times (5, 10, 20, and 30 s) ($P < 0.05$). Inactivation of pathogen in biofilms by SS and SHS treatment is mainly due to latent heat (Huang, 2004). During SS or SHS treatment, the condensation of SS or SHS onto the coupon surface generates a transfer of heat energy (latent heat), which causes rapid heating of the coupon surface, effectively destroying any pathogen biofilms (Castell-Perez and Moreira, 2004).

The coupon temperature profile explains why stainless steel coupons treated with SS or SHS inactivated biofilm cells more rapidly compared to PVC (Fig. 1). Biofilm cells undergo different thermal stresses between stainless steel and PVC coupons, because thermal conductivity of stainless steel (16 W/m·K) is considerably higher than that of PVC (0.19 W/m·K) when heat energy of SS or SHS is transferred to coupon surfaces (Ban et al., 2012).

This supports the results of this study. Thermal conductivity of coupons significantly influenced the thermal inactivation of the three pathogens' biofilm cells. SS or SHS treatment on stainless steel coupons resulted in greater reduction in biofilm cells of three pathogens than those on PVC ones. Stainless steel coupons caused more cellular reduction of about 1.95 log CFU/coupon, averaged from three pathogens' biofilms, than PVC coupons when treated with steam for 10 s at 150 ℃.

Stainless steel is moderately hydrophilic with a negative surface charge (Frank, 2000), while PVC is hydrophobic. The different hydrophobic characteristics of PVC and stainless steel affect bacterial attachment and detachment to surfaces (Adlerberth et al., 1996). If surface tension of the pathogen is higher than that of the surrounding medium, cells tend to attach to hydrophilic (high surface tension) surfaces. In general, bacterial surface tension is lower than that of the surrounding medium and more typically adherence to hydrophobic surfaces is observed (Frank, 2000). Beresford et al. (2001) validated that *L. monocytogenes* adherence was greater on PVC than on stainless steel after a short exposure time and after 2 h incubation, but this difference was not significant. Also, in this study, there was no significant difference in the initial cell population on stainless steel and PVC coupon surfaces. It is thought that factors in addition to surface conditioning, roughness, and micro-topography and hydrophobic interaction, such as electrostatic and exopolymer interactions, affect the attachment of bacteria to various materials (Blackman and Frank, 1996; Palmer et al., 2007). Although

these other factors are important, it appears that biofilm cells on PVC are more difficult to detach and inactivate than those on stainless steel primarily because of stronger hydrophobic interactions between bacteria and PVC surfaces (Djordjevic et al., 2002).

The results also indicated that effectiveness of SS or SHS depends on properties of the target pathogen. *S.* Typhimurium was relatively more sensitive to heat treatment than *E. coli* O157:H7 or *L. monocytogenes.* Similar results were observed by other investigators. Heat tolerance of *E. coli* O157:H7 is regulated by *rpoS*, but not all strains (Ahmed et al., 2005; Cheville et al., 1996). Also, *L. monocytogenes* is relatively tolerant to heat (Skandamis et al., 2008). In this study, *E. coli* O157:H7 was more sensitive to the lethal effect of SS or SHS treatment compared to *L. monocytogenes*. The tolerance of *L. monocytogenes* may be related to the difference between Gram-negative and Gram-positive characteristics of the bacteria. Generally, Gram-positive bacteria are more resistant to heat treatment than Gram-negative bacteria (Smelt, 1998).

In this study, results indicate that SHS treatment is effective for inactivating Gram-positive and heat tolerant foodborne pathogens compared to SS treatment. As SHS treatment temperature rose, reduction of the three pathogens' biofilm cells on coupons increased. For PVC, SHS treatment longer than 30 s was not adequate because these coupons became limp and lost functional property at exposure times longer than 30 s. For this reason, SHS should be applied at a higher temperature for a shorter time in order to

inactivate foodborne pathogen biofilms on PVC. SHS at 175 to 200℃ for 30 s was the optimal inactivation condition in the three pathogens' biofilm cells on PVC and stainless steel, which reduced cell numbers below the detection limit (1.48 log) while maintaining functional property of material. Other researchers have studied the effects of SHS for inactivating pathogens on chicken skin, raw almonds and other foods (Bari et al., 2010; Kondjoyan and Portanguen, 2008). Kondjoyan and Portanguen (2008) reported that SHS was clearly more efficient to inactivate *L. innocua* than non-SHS methods, leading to an average reduction of more than 5 log after 30 s treatment. In the present study, reduced cell numbers below the detection limit occurred when rapid heat transfer accompanied by decreasing temperature when condensation on coupons took place. This was especially pronounced with SHS rather than SS treatment. If SHS contacts surfaces, more latent heat and energy is emitted than with SS because the temperature and enthalpy of SHS is higher than that of SS.

When bacteria are exposed to heat stresses, they activate the expression of more than 20 genes encoding chaperones, proteases, and transcriptional regulators and stabilize heat-denatured proteins through the heat shock-associated proteins, improving the bacterial resistance to environmental challenges such as heat stresses (Chuang and Blattner, 1993). The bacterial heat shock response is implicated not only in heat shock stress, but also in a variety of unfavorable conditions including oxidative stress, high osmolarity, nutrients starvation, and hostile host environments (Groisman and Saier, 1990;

Kusukawa and Yura, 1988; Morgan et al., 1986; Volker et al., 1992). Genes encoding heat shock proteins and general stress resistance proteins showed transcriptional surges after 1 s of SHS treatment at 200℃, with parallel induction of stress-related regulator genes including *rpoE*, *rpoS*, and *rpoH* in my previous study (Ban et al., 2015). Interestingly, *Salmonella* biofilm cells exposed to SHS showed decreased transcription of flagella and *Salmonella* pathogenicity island-1 (SPI-1) genes required for motility and invasion of host cells, respectively, whereas increased transcription of SPI-2 genes, important for bacterial survival and replication inside host cells, was detected (Ban et al., 2015). When the transcriptional response was compared between cells treated with SHS (200℃) and SS (100℃), SHS caused immediate changes in gene expression by shorter treatments (Ban et al., 2015). Understanding the status of *Salmonella* virulence and stress resistance induced by SHS treatments is important for wider application of SHS in controlling *Salmonella* biofilm formation during food production.

In this study, these results reveal that SHS treatment is highly lethal to foodborne pathogen biofilm cells and validate that SHS-mediated sanitation is applicable to food processing facilities to protect products from microbial contamination, while simultaneously saving processing and treating time. This study confirms that SHS is an effective alternative for improving inactivation of biofilm cells of three pathogens on PVC and stainless steel which are commonly encountered on surfaces in food processing facilities. However, in order for SHS to be applied substantially at the food processing industry,

deformation of the PVC at high temperature should be noticed. Statistical analysis between SS and SHS treatments showed that SHS had a stronger biofilm cells-inactivating effect *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* ($P < 0.05$). All food processing facilities are constructed of materials which are subject to the corrosive nature by chemical treatment, furthermore, consumers dislike the use of chemicals in food processing. In this regard, SHS, which avoids the use of chemicals, is an excellent countermeasure that is economically feasible as well as inactivates biofilm cells, saving treatment time for the food processing industry.

Chapter II

Effectiveness of Superheated Steam to Inactivate Foodborne Pathogens on Agricultural Produce

Chapter II(1)

Effectiveness of Superheated Steam for Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Enteritidis PT 30, and *Listeria monocytogenes* on Almonds and Pistachios

II(1)-1 Introduction

In recent years, concerns about foodborne outbreaks involving low water activity (a_w) foods have increased (Scott et al., 2009), because salmonellosis is known to be linked to diverse dry foods such as almonds (Isaacs et al., 2005), peanuts, and peanut butter (CDC, 2009). Salmonellosis causes diarrhea, fever, and abdominal cramps 12 to 72 h after infection (Baird-Parker, 1990; Blaser and Newman, 1982). More recently, *Escherichia coli* O157:H7 illnesses have been epidemiologically linked to consumption of in-shell hazelnuts (FDA, 2011). *E. coli* O157:H7 is a pathogen causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). In 2010 and 2014, walnuts were recalled after isolation of *Salmonella* (FDA, 2010) and *Listeria monocytogenes* (FDA, 2014). *L. monocytogenes* infection results in abortion, encephalitis, febrile gastroenteritis, meningitis, and sepsis (Schlech and Acheson, 2000). Cross contamination of raw almonds can readily occur under typical harvesting, drying, and hulling-shelling practices (CDC, 2004). Furthermore, foodborne pathogens are able to survive in dry environments such as almond kernels and pistachios for prolonged periods of time (Kimber et al., 2012; Uesugi et al., 2006).

To inactivate *Salmonella* on almonds, several methodologies such as propylene oxide fumigation (Danyluk et al., 2005), infrared heat (Brandl et al., 2008), hot oil (Du et al., 2010), high hydrostatic pressure (Willford et al., 2008), acidic sprays (Pao et al., 2006), chlorine dioxide (Wihodo et al., 2005),

and steam (Chang et al., 2010; Lee et al., 2006) have been evaluated. However, a maximum residue limit of propylene oxide fumigant has not been established (Brandl et al., 2008) and chlorine dioxide can lead to discoloration of almond surfaces at high concentrations (Wihodo et al., 2005). In particular, saturated steam (SS) pasteurization increases moisture content of the nuts and thus, requires additional processing to remove excess moisture before storage (Brandl et al., 2008).

Superheated steam (SHS) is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure, and a drop in temperature of SHS will not result in condensation unless the temperature is decreased to below the saturation temperature point corresponding to the processing pressure (Cenkowski et al., 2007). SHS has long been known as a safe, non-polluting technology with low energy consumption (Chou and Chua, 2001). SHS transfers a larger amount of heat to the subject of treatment than SS (James et al., 2000; Topin and Tadrist, 1997). However, the inactivation of foodborne pathogens by SHS has rarely been studied, only for *Salmonella* on almonds (Bari et al., 2010).

Therefore, the purpose of this study was to compare and evaluate the effectiveness of SS and SHS for inactivating four foodborne pathogens on the surface of almonds and in-shell pistachios. Also, the effect of SHS treatment on the quality of almonds and pistachios was determined by measuring the color change, texture, acid value (AV), and peroxide value (PV).

II(1)-2. Materials and methods

2.1. Sample preparation

Raw (untreated) almonds (*Prunus dulcis*), 'Nonpareil' cultivar, used in this study (size 27-30: 27 to 30 kernels per 28 g) were provided by Hilltop Ranch (Ballico, CA). Raw in-shell pistachios (*Pistacia vera*) used in this study were large-sized U.S. Extra number 1 grade, obtained from Setton International Foods inc (Terra Bella, CA).

2.2. Bacterial strains and inoculum preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), *S.* Enteritidis phage type (PT) 30 and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80℃ in 0.7 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37℃ for 24 h, and stored at 4℃.

Before each treatment, bacterial strains were prepared according to the method of Danyluk et al. (2005) with minor modifications. For growth experiments, the inoculum consisted of stationary phase cells that were obtained by inoculating TSB with a single colony from a TSA plate and

incubating at 37℃ for 24 ± 2 h. A loop of this culture was transferred into TSB and incubated at 37℃ for 18 ± 2 h to ensure healthy cell growth. This overnight culture (1 ml) was spread onto TSA plates which were then incubated at 37℃ for 24 ± 2 h to produce a bacterial lawn. Four plates were prepared per 400 g almond and pistachio samples. Following incubation, bacterial cells were collected with a sterile cotton swab and suspended in 25 ml of 0.2% peptone water. The cell suspensions were pooled and thoroughly mixed for 1 min with a magnetic stir bar and stir plate. Inoculum levels were determined by tenfold serial dilution in 0.2% peptone water and spread plating the inoculum onto TSA and Xylose Lysine Desoxycholate agar (XLD; Difco). Plates were incubated at 37℃ for 24 ± 2 h and colonies characteristic of *Salmonella* spp. were enumerated.

2.3. Inoculation procedure

Each almond (400 g) and in-shell pistachio sample was weighed into a plastic polyethylene bag (30 × 25 cm) and 25 ml of inoculum was added. The bag was sealed and thoroughly mixed by hand massaging for 60 s. Almonds and pistachios were poured out of the bag and spread onto filter paper and dried overnight at room temperature (22 ± 2℃)

2.4. Saturated steam and superheated steam treatment

SS at 100℃, produced by a SS generator, was introduced into a SHS steam generator through a flexible tube. SS was converted into SHS by

heating with an electrical resistance heater in the SHS generator. The maximum temperature of SHS generated in this study was about 200℃. During these experiments, the SS and SHS temperature was controlled automatically by means of a temperature sensor and intelligent power module in each of the steam generators.

Dried inoculated almonds and pistachios were spread into a single layer on a stainless steel treatment grid and placed in an insulated steam treatment chamber (external diameter 23 cm; external height, 32 cm; internal diameter, 17 cm; internal height, 22.5 cm). A valve placed on top of the treatment chamber was used to control steam flow. Steam passed through the flexible hose and chamber by opening the steam valve. Almonds and pistachios were steam treated for 1, 5, 10, 15, and 20 s and 1, 5, 10, 20, and 30 s, respectively. SS treatment was performed at 100℃ while SHS treatments were performed at 125, 150, 175, and 200℃. The basket was immediately removed from the chamber after each treatment, and almonds or pistachios were then placed in a stomacher bag (Labplas Inc., Sainte-Julie, Quebec, Canada).

2.5. Bacterial enumeration

Treated almond kernels and pistachios were placed in stomacher bags along with 50 ml of 0.2% peptone water. Almond samples were homogenized for 2 min with a mechanical stomacher (EASY MIX, AES Chemunex, Rennes, France). Pistachio samples were shaken for 30 s, rubbed by hand for 15 s, and then shaken for an additional 30 s. After homogenization, 1 ml aliquots of

samples were 10-fold serially diluted with 9 ml of sterile 0.2% peptone water, and 100 µl of appropriate dilutions were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate surviving populations of *E. coli* O157:H7, *S.* Typhimurium and *S.* Enteritidis PT 30, and *L. monocytogenes*, respectively. When low bacterial numbers were anticipated, 1 ml was distributed over four petri dishes (0.25 ml each). As a control (time-zero survival), untreated almonds and pistachios inoculated with the four pathogens were stomached and shaken, respectively, diluted and plated. All plates were incubated at 37℃ for 24 h, and then colonies enumerated. To confirm pathogen identity, presumptive colonies were randomly selected from selective media and subjected to biochemical and serological tests. These tests consisted of the *E. coli* O157:H7 latex agglutination assay (Oxoid, Basingstoke, UK), the *Salmonella* latex agglutination assay (Oxoid, Basingstoke, UK), and the API Listeria test (BioMérieux, Hazelwood, MO).

2.6. Color and texture measurement

Color assessments were measured using a Minolta colorimeter (Model CR-400; Minolta Camera Co. Ltd., Osaka, Japan). Measurements were taken from SS and SHS treated and untreated samples measured at random locations on almonds and pistachios and averaged. L* (intensity of lightness), a* (intensity of redness), and b* (intensity of yellow color) values were measured

in triplicate for each treatment.

Changes in texture of SS and SHS treated almonds and pistachios were evaluated with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set probe. After treated samples were dried, a sample was placed onto the press holder, and a blade was moved down at 2 mm/s. Maximum force was recorded using TexturePro CT software (Brookfield Engineering Laboratories, Inc.). Three measurements were performed for each treatment with independently-prepared samples.

2.7. Acid value and peroxide value

Indicators of lipid oxidation in SS and SHS treated almonds and pistachios were measured by AV and PV. AV and PV were determined using official methods Cd 3d-63 and 8b-90 of the American Oil Chemists' Society (AOAC, 1998, 2007). Analyses were done in triplicate.

2.8. Statistical analysis

All experiments were repeated three times with duplicate samples. Triplicate data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple-range test at a probability level of $P < 0.05$.

II(1)-3. Results

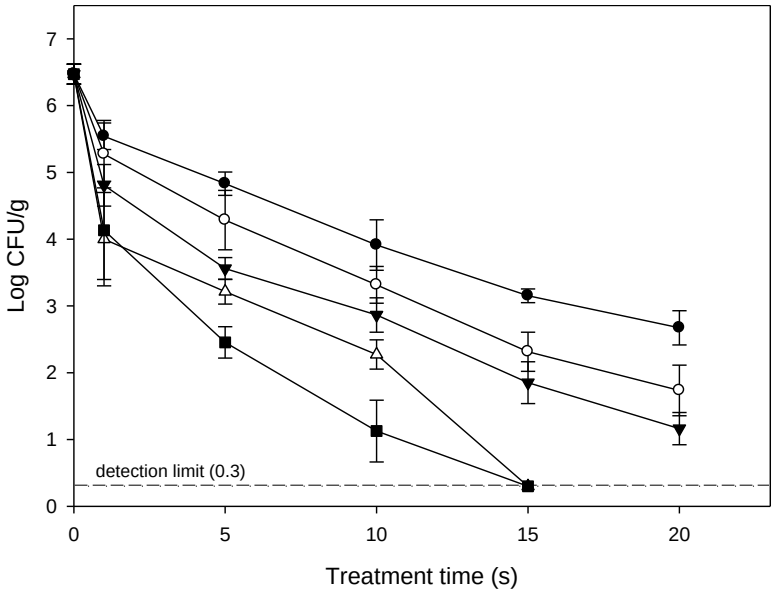
3.1. Inactivation of pathogenic bacteria on almonds

Survival (log CFU/g) of *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes* on almonds and pistachios after SS and SHS treatment are shown in Fig. 1 and 2. Initial inoculum levels of *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes* on almonds were 6.47, 6.84, 6.96, and 6.05 log CFU/g and those on pistachios were 6.42, 6.56, 6.69, and 5.82 log CFU/g, respectively. Significant ($P < 0.05$) log reductions of the four pathogens were observed as the duration of SHS treatment increased. SHS treatment at 200℃ for 15 s achieved 6.17, 6.54, 6.66, and 5.75 log reductions in *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes* on almonds, respectively, whereas SS treatment at 100℃ attained 3.91, 3.76, 2.89, and 2.39 log reductions for each pathogen. Levels of the four pathogens on pistachios were reduced by 6.12, 6.26, 6.39, and 5.52 log for *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes*, respectively, after SHS treatment at 200℃ for 30 s, whereas these pathogens experienced log reductions of 3.04, 3.26, 2.73, and 2.70 after SS treatment at 100℃ for the same time interval. It was observed that SHS treatment caused an additional 1.84–4.15 and 2.05–3.56 log reductions of the three pathogens on almonds and pistachios, respectively, compared to SS treatments. The populations of the four pathogens on almonds were reduced to below the detection limit when

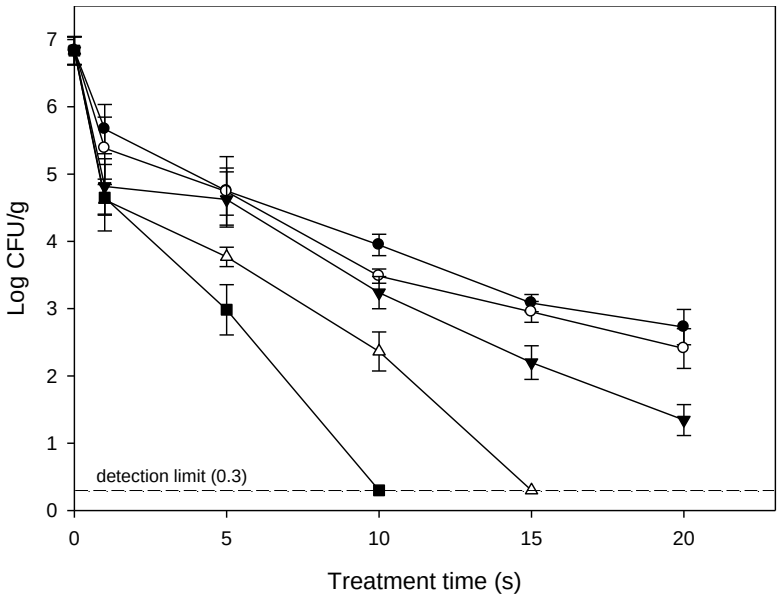
subjected to heating for 15 s at 175℃ and for 10 s at 200℃, for 15 s at 175 and 200℃, for 15 s at 200℃, and for 20 s at 175℃ and for 15 s at 200℃ for *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes*, respectively.

Fig. 2 shows that the overall reduction tendencies of *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes* on pistachios were similar to those on almonds. However, a longer treatment time was needed for pistachios than for almonds to reduce populations to below the detection limit. The four pathogens on pistachios were fully inactivated (reduced to below the detection limit) when treated for 30 s at 175 and 200℃, for 30 s at 175℃ and for 20 s at 200℃, for 30 s at 200℃, and for 30 s at 200℃ for *E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis PT 30, and *L. monocytogenes*, respectively.

A


Log CFU/g
Treatment time (s)
detection limit (0.3)
0
1
2
3
4
5
6
7
0
5
10
15
20

B


Log CFU/g
Treatment time (s)
detection limit (0.3)
0
1
2
3
4
5
6
7
0
5
10
15
20

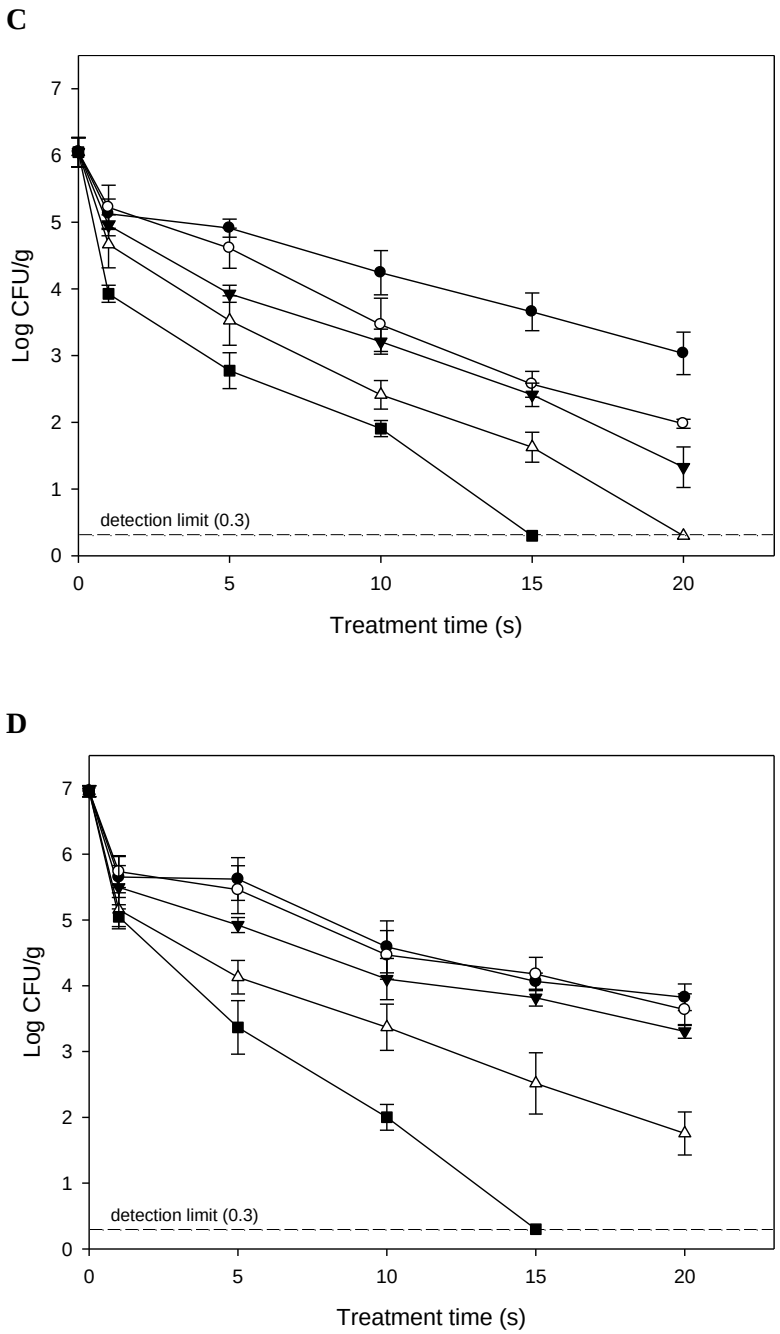
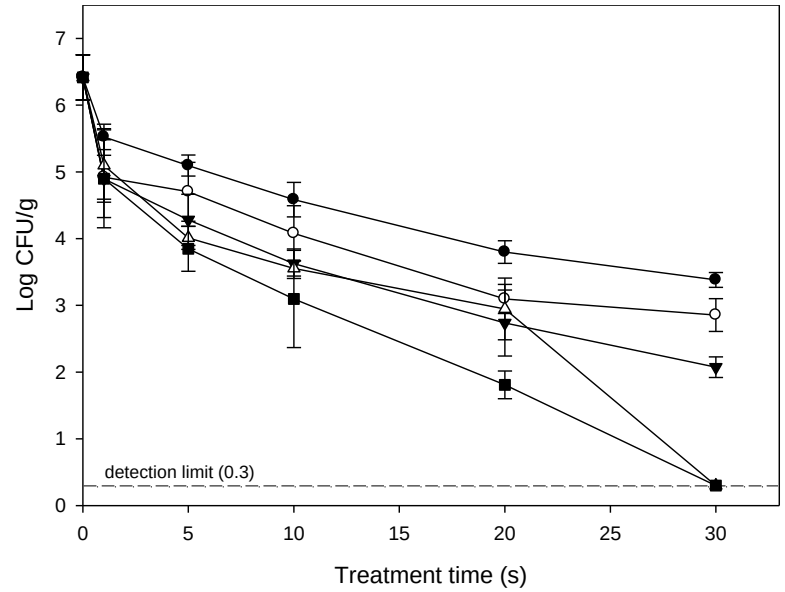
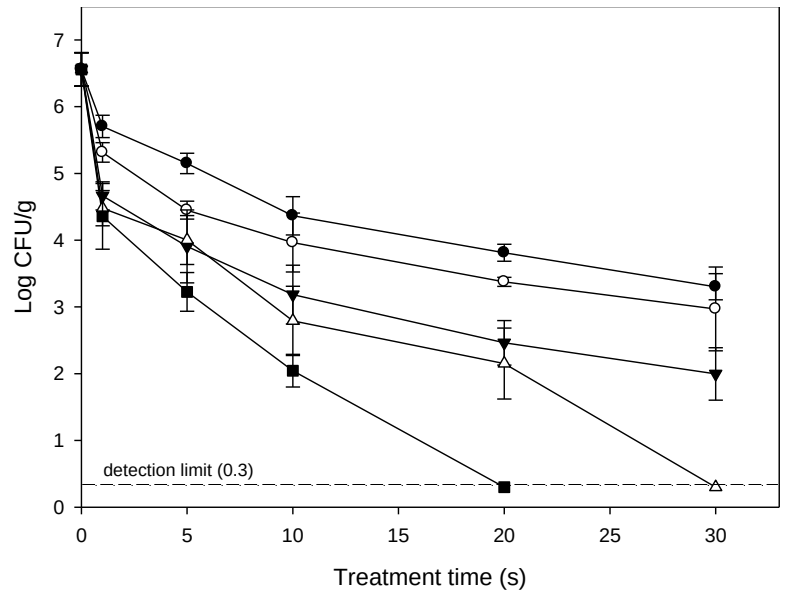


Fig. II(1)-1. Survival curves for *Escherichia coli* O157:H7 (A), *Salmonella* Typhimurium (B), *Listeria monocytogenes* (C), and *Salmonella* Enteritidis PT 30 (D) on almonds treated with SS at 100°C (●), SHS at 125°C (○), SHS at 150°C (▼), SHS at 175°C (△), SHS at 200°C (■).

A


Log CFU/g
Treatment time (s)
detection limit (0.3)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30

B


Log CFU/g
Treatment time (s)
detection limit (0.3)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30

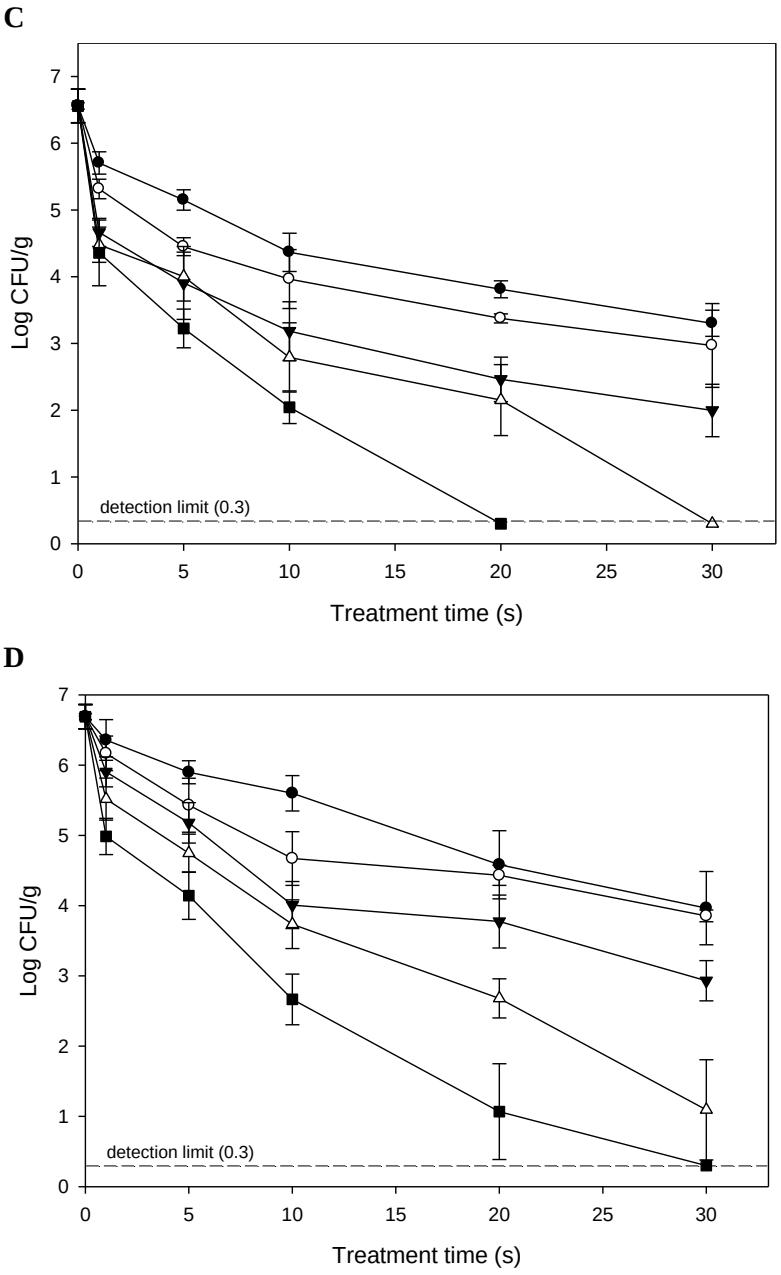


Fig. II(1)-2. Survival curves for *Escherichia coli* O157:H7 (A), *Salmonella* Typhimurium (B), *Listeria monocytogenes* (C), and *Salmonella* Enteritidis PT 30 (D) on pistachios treated with SS at 100°C (●), SHS at 125°C (○), SHS at 150°C (▼), SHS at 175°C (△), SHS at 200°C (■).

3.2. Effect of SS and SHS treatment on color and texture of almonds and pistachios

The color values of almonds and pistachios after SS and SHS treatment are shown in Table 1. The L*, a*, and b* values of SS and SHS treated almonds and pistachios were not significantly ($P > 0.05$) different from those of untreated controls. Table 2 shows the texture parameters of almonds and pistachios following SS and SHS treatment. There were no significant ($P > 0.05$) differences between maximum load values of texture measurements among all tested samples, indicating that treatment with SHS at 200℃ for 15 s and 30 s did not significantly ($P > 0.05$) change the quality of almonds and pistachios, respectively.

Table II(1)-1. Color analysis of steam treated almonds (A) and pistachios (B) where L* is lightness, a* is redness, and b* is yellowness

(A) Almonds						
	Treatment time (s)					
L*						
Temperature (℃)	0	1	5	10	15	20
25	48.40 ± 1.42 A	-	-	-	-	-
100	-	48.43 ± 0.46 A	47.98 ± 0.42 A	48.54 ± 0.38 A	48.64 ± 2.76 A	48.30 ± 1.11 A
125	-	48.73 ± 0.43 A	48.39 ± 0.62 A	48.25 ± 0.37 A	48.66 ± 1.29 A	48.24 ± 0.95 A
150	-	48.38 ± 1.10 A	48.24 ± 0.89 A	48.33 ± 0.71 A	48.59 ± 1.01 A	48.57 ± 0.40 A
175	-	48.28 ± 1.03 A	48.17 ± 0.46 A	48.63 ± 0.47 A	48.44 ± 0.89 A	48.68 ± 0.57 A
200	-	48.52 ± 0.62 A	48.43 ± 0.49 A	48.45 ± 1.14 A	47.69 ± 0.54 A	48.34 ± 0.77 A
a*						
25	8.23 ± 0.58 A	-	-	-	-	-
100	-	8.51 ± 0.38 A	8.26 ± 0.25 A	8.27 ± 0.24 A	8.43 ± 0.27 A	8.56 ± 0.36 A
125	-	8.13 ± 0.21 A	8.60 ± 0.19 A	8.56 ± 0.36 A	8.27 ± 0.06 A	8.20 ± 0.24 A
150	-	8.24 ± 0.26 A	8.30 ± 0.19 A	8.34 ± 0.22 A	8.35 ± 0.53 A	8.54 ± 0.16 A
175	-	8.61 ± 0.25 A	8.27 ± 0.56 A	8.69 ± 0.33 A	8.27 ± 0.24 A	8.45 ± 0.27 A
200	-	8.49 ± 0.43 A	8.45 ± 0.33 A	8.66 ± 0.28 A	8.10 ± 0.22 A	8.31 ± 0.49 A
b*						
25	0.41 ± 0.07 A	-	-	-	-	-
100	-	0.40 ± 0.02 A	0.40 ± 0.03 A	0.39 ± 0.02 A	0.42 ± 0.03 A	0.44 ± 0.03 A
125	-	0.40 ± 0.02 A	0.39 ± 0.01 A	0.40 ± 0.01 A	0.42 ± 0.04 A	0.42 ± 0.02 A
150	-	0.38 ± 0.04 A	0.42 ± 0.04 A	0.43 ± 0.03 A	0.44 ± 0.03 A	0.41 ± 0.03 A
175	-	0.41 ± 0.02 A	0.41 ± 0.05 A	0.41 ± 0.02 A	0.41 ± 0.02 A	0.43 ± 0.04 A
200	-	0.43 ± 0.04 A	0.41 ± 0.05 A	0.41 ± 0.03 A	0.40 ± 0.01 A	0.43 ± 0.06 A

(B) Pistachios

	Treatment time (s)					
L*						
25	53.57 ± 2.35 A	-	-	-	-	-
100	-	53.24 ± 1.85 A	54.05 ± 1.57 A	53.18 ± 1.32 A	54.32 ± 0.96 A	53.90 ± 0.91 A
125	-	53.42 ± 0.77 A	54.32 ± 1.77 A	53.94 ± 1.36 A	54.17 ± 0.94 A	54.09 ± 1.65 A
150	-	52.71 ± 1.56 A	54.44 ± 1.57 A	54.41 ± 2.75 A	53.50 ± 1.85 A	53.06 ± 1.03 A
175	-	54.41 ± 1.74 A	54.10 ± 1.88 A	52.95 ± 1.34 A	53.78 ± 1.50 A	53.05 ± 1.30 A
200	-	53.77 ± 1.89 A	53.43 ± 2.01 A	53.74 ± 1.97 A	54.27 ± 1.22 A	54.88 ± 1.61 A
a*						
25	3.40 ± 1.57 A	-	-	-	-	-
100	-	2.79 ± 0.48 A	1.75 ± 0.40 A	3.23 ± 1.53 A	4.10 ± 0.95 A	2.94 ± 1.11 A
125	-	3.47 ± 0.47 A	2.95 ± 1.64 A	2.92 ± 0.64 A	2.46 ± 1.38 A	2.77 ± 0.98 A
150	-	2.74 ± 1.52 A	3.71 ± 1.22 A	4.27 ± 0.06 A	3.23 ± 0.34 A	2.78 ± 1.36 A
175	-	3.54 ± 1.25 A	2.90 ± 1.70 A	2.46 ± 1.07 A	3.06 ± 1.17 A	3.19 ± 1.34 A
200	-	3.30 ± 0.78 A	3.46 ± 1.07 A	3.77 ± 1.82 A	3.60 ± 1.57 A	3.29 ± 0.66 A
b*						
25	15.08 ± 2.58 A	-	-	-	-	-
100	-	13.69 ± 1.13 A	14.37 ± 1.69 A	15.35 ± 1.84 A	14.30 ± 1.28 A	14.58 ± 2.28 A
125	-	13.60 ± 2.63 A	15.37 ± 1.98 A	15.33 ± 0.01 A	14.23 ± 0.60 A	13.79 ± 2.29 A
150	-	15.23 ± 1.98 A	13.45 ± 2.23 A	15.68 ± 0.03 A	12.67 ± 0.39 A	15.22 ± 0.78 A
175	-	15.66 ± 2.23 A	14.39 ± 1.24 A	15.40 ± 0.02 A	16.16 ± 2.81 A	16.66 ± 1.49 A
200	-	14.70 ± 1.01 A	16.13 ± 1.01 A	16.20 ± 0.03 A	14.80 ± 0.60 A	14.14 ± 2.67 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

Table II(1)-2. Hardness for texture of almonds (A) and pistachios (B) following treatment with SS and SHS

(A) Almonds

	Treatment time (s)					
Temperature (℃)	0	1	5	10	15	20
25	17.09 ± 2.06 A	-	-	-	-	-
100	-	17.37 ± 1.17 A	17.40 ± 0.84 A	15.70 ± 1.28 A	16.03 ± 2.07 A	17.34 ± 1.95 A
125	-	17.86 ± 0.74 A	17.58 ± 0.16 A	16.67 ± 0.31 A	17.68 ± 0.96 A	17.32 ± 0.59 A
150	-	15.18 ± 2.09 A	17.21 ± 0.80 A	17.79 ± 0.91 A	17.30 ± 0.20 A	16.98 ± 1.04 A
175	-	17.38 ± 1.34 A	18.37 ± 0.81 A	16.36 ± 0.95 A	17.30 ± 1.03 A	16.99 ± 0.87 A
200	-	16.80 ± 1.42 A	16.89 ± 0.58 A	15.64 ± 0.77 A	16.46 ± 1.28 A	16.56 ± 1.26 A

(B) Pistachios

	0	1	5	10	20	30
25	13.60 ± 1.46 A	-	-	-	-	-
100	-	13.73 ± 0.99 A	15.15 ± 1.27 A	14.51 ± 1.74 A	13.06 ± 0.66 A	13.63 ± 0.73 A
125	-	15.29 ± 1.76 A	15.35 ± 1.80 A	13.75 ± 1.25 A	14.67 ± 1.40 A	14.43 ± 0.88 A
150	-	12.49 ± 0.38 A	14.39 ± 0.90 A	15.06 ± 1.01 A	14.80 ± 1.74 A	14.02 ± 1.31 A
175	-	15.34 ± 2.63 A	14.65 ± 1.55 A	14.15 ± 0.88 A	13.73 ± 0.53 A	14.08 ± 1.09 A
200	-	15.92 ± 2.87 A	13.44 ± 0.82 A	14.84 ± 0.22 A	13.56 ± 0.64 A	14.34 ± 1.03 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

3.3. Effect of SS and SHS treatment on lipid oxidation of almonds and pistachios

Changes of oxidative rancidity of SS and SHS treated almonds and pistachios are shown in Table 3 and 4. There was a slight difference in AV between samples treated with SS and SHS compared to controls. After 1 s of SS and SHS heating, no significant ($P > 0.05$) differences between samples were found as temperature increased. After SS or SHS exposure for 20 s and 30 s on almonds and pistachios, respectively, there were significant differences between the untreated and treated samples in AV and PV ($P < 0.05$). The PV of almonds and pistachios treated with SS and SHS for 20 s and 30 s, respectively, decreased compared to untreated samples.

Table II(1)-3. Acid values of almond (A) and pistachio (B) after exposure to SS and SHS for a range of treatment times

(A) Almonds

	Treatment time (s)					
Temperature (℃)	0	1	5	10	15	20
25	0.34 ± 0.03 A	-	-	-	-	-
100	-	0.34 ± 0.02 A	0.36 ± 0.03 A	0.34 ± 0.02 A	0.34 ± 0.02 A	0.30±0.01 AB
125	-	0.33 ± 0.01 A	0.35 ± 0.01 A	0.36 ± 0.02 A	0.35 ± 0.03 A	0.29±0.01 B
150	-	0.34 ± 0.03 A	0.35 ± 0.03 A	0.35 ± 0.01 A	0.34 ± 0.02 A	0.29±0.02 AB
175	-	0.35 ± 0.03 A	0.35 ± 0.01 A	0.35 ± 0.03 A	0.31 ± 0.01 AB	0.29±0.02 AB
200	-	0.35 ± 0.03 A	0.34 ± 0.03 A	0.34 ± 0.01 A	0.31 ± 0.02 AB	0.29±0.01 B

(B) Pistachios

	0	1	5	10	20	30
25	0.54±0.02 A	-	-	-	-	-
100	-	0.53±0.02 A	0.52±0.02 A	0.53±0.03 A	0.56±0.04 A	0.53±0.02 A
125	-	0.53±0.02 A	0.50±0.01 B	0.53±0.03 A	0.55±0.01 A	0.55±0.04 A
150	-	0.57±0.03 A	0.53±0.02 A	0.53±0.04 A	0.55±0.02 A	0.53±0.02 A
175	-	0.53±0.06 A	0.52±0.03 A	0.51±0.02 A	0.55±0.04 A	0.55±0.01 A
200	-	0.55±0.06 A	0.53±0.01 A	0.52±0.04 A	0.50±0.02 B	0.57±0.01 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

Table II(1)-4. Peroxide (meq/kg) values of almonds (A) and pistachios (B) after exposure to SS and SHS for a range of treatment times

(A) Almonds

	Treatment time (s)					
Temperature (℃)	0	1	5	10	15	20
25	0.24 ± 0.02A	-	-	-	-	-
100	-	0.22 ± 0.01 A	0.22 ± 0.02 AB	0.21 ± 0.02 AB	0.22 ± 0.01 A	0.19 ± 0.01 B
125	-	0.24 ± 0.02 A	0.23 ± 0.01 A	0.21 ± 0.02 AB	0.21 ± 0.01 AB	0.20 ± 0.02 AB
150	-	0.23 ± 0.01 A	0.23 ± 0.01 A	0.21 ± 0.02 AB	0.21 ± 0.02 AB	0.20 ± 0.02 AB
175	-	0.22 ± 0.01 A	0.24 ± 0.02 A	0.22 ± 0.01 A	0.20 ± 0.01 AB	0.19 ± 0.01 B
200	-	0.24 ± 0.02 A	0.23 ± 0.01 A	0.21 ± 0.01 AB	0.21 ± 0.01 AB	0.19 ± 0.01 B

(B) Pistachios

	0	1	5	10	20	30
25	0.25 ± 0.03 A	-	-	-	-	-
100	-	0.24 ± 0.02 A	0.24 ± 0.02 A	0.25 ± 0.02 A	0.25 ± 0.02 A	0.21 ± 0.01 AB
125	-	0.24 ± 0.02 A	0.25 ± 0.03 A	0.25 ± 0.02 A	0.22 ± 0.01 AB	0.21 ± 0.01 AB
150	-	0.25 ± 0.02 A	0.24 ± 0.02 A	0.23 ± 0.02 AB	0.22 ± 0.02 AB	0.20 ± 0.01 B
175	-	0.25 ± 0.03 A	0.25 ± 0.01 A	0.25 ± 0.01 A	0.24 ± 0.02 A	0.19 ± 0.02 B
200	-	0.25 ± 0.01 A	0.24 ± 0.02 A	0.23 ± 0.01 A	0.22 ± 0.02 AB	0.19 ± 0.02 B

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

II(1)-4. Discussion

Foodborne pathogens on tree nuts have been linked to large-scale outbreaks and are responsible for the recall and destruction of large quantities of nut products. This study has evaluated the effectiveness of SHS against four pathogens on almonds and pistachios to assess its ability to control pathogens on nuts.

SHS heating has proven to be one of the most useful drying methods for biological or non-biological products, such as foods (Braud et al., 2001), wood chips (Johansson et al., 2008), or even porous materials (Hager et al., 1997). Furthermore, SHS combined with impingement technology, which enhances the strength of both steam blanching and impingement technology, has resulted in a uniform, rapid, energy-efficient blanching process (Xiao et al., 2014).

In recent years, investigations of inactivation efficiency utilizing SHS have been performed by a few researchers. Kondjoyan and Portanguen (2008) reported that SHS was distinctly more efficacious for inactivating *Listeria innocua* than non-SHS, leading to an average reduction of more than 5 log after 30 s treatment. Bari et al. (2010) conducted a SHS and gas catalytic infrared heat treatment study to inactivate *Salmonella* on raw almonds. SHS treatment for 70 s followed by catalytic infrared heat treatment for 70 s was able to reduce *Salmonella* populations by 5.73 log CFU/g (below the detection limit). Furthermore, no *Salmonella* was detected in the enrichment medium,

suggesting that *Salmonella* cells were completely inactivated. Our previous study showed that SHS treatment effectively reduced populations of biofilm cells and reduced disinfection time compared to SS treatments (Ban et al., 2014). In this study, the numbers of biofilm cells were reduced to below the detection limit (1.48 log CFU/coupon), after exposure to 200℃ steam for 30 s or 10 s on PVC or stainless steel, respectively. In the present study, foodborne pathogens on almonds and pistachios were fully inactivated after 200℃ steam treatment for 15 s and 30 s, respectively.

In the study of inactivation of biofilm cells by SHS, cells were exposed to different thermal stresses between stainless steel and PVC coupons, because thermal conductivity (*k*) of stainless steel (16 W/m·K) is much higher than that of PVC (0.19 W/m·K) when heat energy of SS or SHS is transferred to coupon surfaces (Ban et al., 2012). However, in the present study, thermal conductivity of the two types of nuts did not influence the inactivation of foodborne pathogens because their *k* values are similar (almonds, 0.64 W/m·K; pistachios, 0.66 W/m·K) (Choi and Okos, 1986). It seems to be more difficult for steam to penetrate beneath the shells for inactivating foodborne pathogens on pistachio kernels compared to almonds which have no shells to interfere. For this reason, bacterial populations on pistachios did not receive the same thermal effect as on almonds. Although their exterior features are different, foodborne pathogens on pistachios within the shell were fully inactivated as well as on almonds.

Bacteria are very small and can survive in the cracks, crevices, and pores of almonds and pistachios which can prevent their coming into contact with several control agents such as hot air and hot water. Steam is known to effectively penetrate cavities, crevices, and feather follicles that may provide protection for surface attached microorganisms against water because of the high surface tension of aqueous fluids (Morgan et al., 1996). Lee et al. (2006) observed that 35 s of 93℃ steam treatment resulted in a 3.8 log reduction of *Salmonella* on 'Nonpareil' almonds and 61 s of steam duration would be required to attain 5 log reductions. Although steam treatment for 30 to 40 s effectively reduced *Salmonella* populations in almonds, the moisture content increased during the treatment time. Chang et al. (2010) ascertained that a 5 log reduction of *S.* Enteritidis was achieved following steam treatment at 95℃ for 25 s. However, visual quality degradation including detachment of almond skin, wrinkled almond surfaces and the presence of small grey spots on almond skin were observed after a 35 s steam pasteurization treatment. In our study, the same phenomenon was observed, especially after 100℃ SS treatment (data not shown). Moisture addition results in quality loss caused by loosened skin and increased mold growth as well as added costs for removing excess moisture from SS treated almonds (Almond Board of California, 2003).

In the present study, four foodborne pathogens on almonds and pistachios were fully inactivated after SHS treatment for 20 and 30 s, respectively. And, in a preliminary study, moisture content was not affected by 200℃ SHS treatment compared to SS treatment (3.77, 5.28, and 4.08% for untreated, SS

at 100℃, and SHS at 200℃ for almonds, respectively; 4.72, 5.97, and 5.16% for untreated, SS at 100℃, and SHS at 200℃ for pistachios, respectively). Unlike SS, a drop in SHS temperature during processing will not result in condensation of steam, as long as the temperature is still higher than the saturation temperature at the processing pressure (Pronyk et al., 2004). SHS drying has been used for various dried foods including flour, paprika powder, and onion powder (van Deventer and Heijmans, 2001). These research results imply that SHS treatment can be used as a pasteurization technology for almonds and pistachios without the need to be concerned about increasing moisture content.

In previous almond and pistachio pasteurization studies, evaluations of quality changes were limited to subjective visual observations and statistical analyses were rarely conducted. For this reason, our experimental conditions focused on maintaining the quality of almonds and pistachios after SHS treatment compared to untreated samples. After SHS treatment for up to 20 and 30 s on almonds and pistachios, respectively, color values and maximum load values were not significantly ($P > 0.05$) different from untreated controls. Although L*-values (lightness) were not consistent, significant differences in color measurements were not observed. And, general observations by the experimental team were not able to detect color changes between the samples. The PV of SS or SHS treated samples decreased compared to untreated samples. Hot air and RF treatments did not significantly affect the PV and free fatty acids of almonds when measured immediately after treatment (Gao et al.,

2010). The mean PV of hot air and RF treated almonds after 10 and 20 day storage periods were lower than those of untreated controls (Gao et al., 2010). This might be due to possible inactivation of lipoxygenase enzymes by heat treatments (Buranasompob et al., 2001). Lipoxygenase in dry pinto beans lost all activity after 15 s at 100℃ and 93% of their initial activity after 10 min at 65℃ (McCurdy et al., 1983). For both almonds and pistachios, the AV and PV after SS and SHS treatment for up to 15 s and 30 s, respectively, fell within the acceptable range (PV < 1.0 meq/kg) for good quality used by industry. Thus, short time heat treatment of almonds and pistachios did not promote rancidity. These results show that thermal application of 200℃ SHS treatment for 15 s and 30 s, respectively, did not affect the quality of almonds and pistachios.

In conclusion, this research demonstrated that SHS treatment leads to effective inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on almonds and pistachios, as well as preventing quality deterioration. SHS treatment does not need to be combined with other methods to enhance the effectiveness of pasteurization, whereas SS treatment cannot be enhanced by combining it with other methods in an effort to improve efficiency of pasteurization while simultaneously protecting against increasing moisture content, a problem inherent with SS treatment. Therefore, SHS treatment is a very promising alternative technology for the tree nut industry by improving inactivation of foodborne pathogens on almonds and pistachios while reducing processing time and expense.

Chapter II(2)

Effectiveness of Superheated Steam for Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on Cherry Tomatoes and Oranges

II(2)-1. Introduction

Consumption of fresh produces has increased rapidly as consumers are becoming increasingly aware of health and nutrition (Heaton and Jones, 2008). Concomitant with increased consumption of fresh produce come increasing frequency of foodborne disease outbreaks (Sivapalasingam et al., 2004). Fresh produce can become contaminated with foodborne pathogens while growing in fields, orchard, vineyards, or greenhouses, or during harvesting, post-harvest handling, and processing (Beuchat, 1996; 2002). For this reason, controlling foodborne pathogens on fruits and vegetables becomes important to ensure microbial safety and promote consumer health.

Escherichia coli O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* have been implicated in foodborne illness outbreaks involving consumption of fresh fruits and vegetables (Beuchat, 1996; Little and Gillespie, 2008; Sivapalasingam et al., 2004). *E. coli* O157:H7 is an important pathogen capable of causing bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991; Griffin and Tauxe, 1991). *S.* Typhimurium a commonly isolated *Salmonella* serotype, has been implicated in foodborne illnesses characterized by diarrhea, abdominal pain, fever, chills, nausea, and vomiting (Baird-Parker, 1990). Listeriosis caused by *L. monocytogenes* can lead to abortion, neonatal death, septicemia, and meningitis (Schlech and Acheson, 2000)

To sanitize fresh produce, washing with chlorinated water has widely been used on a commercial scale to reduce the microbial load (Parish et al., 2003; Weissinger et al., 2000). However, this treatment produces an antimicrobial effect of less than 2 log CFU/g on fresh fruits and vegetables (Beuchat, 1999; Taormina and Beuchat, 1999) and is known to adversely react with organic matter, resulting in the formation of carcinogenic halogenated by-products (Hua and Reckhow, 2007). Furthermore, continuous exposure to chlorine-based sanitizers has the effect of increasing resistance of microorganisms (Davidson and Harrison, 2002). Furthermore, consumers prefer that fresh produce not be treated with chemicals. Therefore, an alternative new method is needed to effectively reduce pathogens and simultaneously reduce or eliminate chemical use while still maintaining quality.

Recently, research with superheated steam (SHS) treatment has been evaluated for inactivating foodborne pathogens on chicken skin (Kondjoyan and Portanguen, 2008), almonds (Bari et al., 2010), and biofilms on stainless steel and polyvinyl chloride (Ban et al, 2014). SHS is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure (Cenkowski et al., 2007) and has been known as a safe and non-polluting technology with low energy consumption (Chou and Chua, 2001). SHS has various advantages over other heating systems, including a high heat transfer rate due to condensation and gas radiation, accelerated drying rate, and an oxygen-free environment (Bari et al., 2010). SHS treatment is able to transfer a large amount of latent heat to food when steam

condenses on food surfaces because the low initial temperature of a food surface rapidly increases (Iyota et al., 2001, James et al., 2000). Condensation of SHS occurrs on food surfaces and then the condensed water evaporates back into the SHS because of its low moisture content, which leads to drying of the surface (Iyota et al., 2001). Although several researchers observed that SHS heating has a strong killing effect against foodborne pathogens, there have been no studies to demonstrate the inactivation effect of SHS on fresh produce. Because fresh produce is sensitive to heat, quality changes following thermal treatment with SS and SHS needs to be assessed.

Therefore, the purpose of this study was to compare and evaluate the bactericidal effectiveness of SS and SHS on cherry tomatoes and oranges, and investigate the quality changes following treatment with SS and SHS. To be specific, we evaluated the possibility of using SHS treatment on fresh produce and determined optimized treatment conditions to ensure both microbial safety and quality of food stuffs.

II(2)-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80℃ in 0.7 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37℃ for 24 h, and stored at 4℃. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37℃ for 24 h. Cells of each strain were collected by centrifugation at 4000 g at 4℃ for 20 min and washed three times with buffered peptone water (BPW; Difco, Sparks, MD). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7–10^8 CFU/ml. Suspended pellets of the three strains of each species were combined to produce a mixed culture cocktail.

2.2. Sample preparation and inoculation procedure

Fresh unblemished and uncoated cherry tomatoes and Valencia oranges were supplied by a local market (Seoul, Korea) on the day before the experiment and stored at 4℃ prior to use. The cherry tomatoes and oranges

were 3 ± 0.5 cm and 8.5 ± 0.5 cm in diameter and 12 ± 1 g and 100 ± 5 g, respectively (n = 100).

Cherry tomatoes and oranges were placed on sterile aluminum foil in a laminar flow hood and spot-inoculated with 0.1 ml of culture cocktail by depositing droplets with a micropipette at 10 to 15 locations. Spot inoculation is more consistent and ensures more reproducible results for inoculation with a known number of pathogen cells adhering to produce surfaces than does the dipping or spraying inoculation methods (Beuchat et al., 2001). Inoculated cherry tomatoes and oranges were then air-dried for 1 h in the hood with the fan running at 22 ± 2℃.

2.3. Saturated steam and Superheated steam treatment

The experimental apparatus consisted of a saturated steam generator with a maximum power of 5 kW at a 220 V input, a superheated steam generator with a maximum power of 6 kW at a 220 V input and a maximum temperature of 200℃, an insulated sample treatment stainless steel chamber (external diameter, 23 cm; external height, 32 cm; internal diameter, 17 cm; internal height, 22.5 cm), and a flexible stainless steel connection hose (Fig.III-1). SS at 100℃, produced by the SS generator, was introduced into the SHS steam generator through a flexible tube. SS was converted into SHS by heating with an electrical resistance heater in the SHS generator. The maximum temperature generated from the SHS generator used in this study was 200℃, which was made by giving additional heat to SS at a 1 atm.

During these experiments, the SS and SHS temperature was controlled automatically by means of a temperature sensor and an intelligent power module (IPM) in each of the steam generators. After SS and SHS temperature of the inlet into the chamber had stabilized (following 5 min warm-up time), two inoculated cherry tomatoes or an inoculated orange were placed on a stainless steel treatment grid (9 by 9 by 10 cm). A valve placed on top of the treatment chamber was used to control steam flow. Steam passed through the flexible hose into the chamber by opening the steam valve. Cherry tomatoes and oranges were steam treated for 1, 2, 3, 4, and 5 s and 1, 5, 10, 20, and 30 s, respectively. SS treatment was performed at 100℃ while SHS treatments were performed at 125, 150, 175, and 200℃. Cherry tomato or orange samples were immediately removed from the chamber after each treatment and placed in a stomacher bag (Labplas Inc., Sainte-Julie, Quebec, Canada) with 225 or 50 ml of 0.2% peptone water, respectively, and put in ice water to prevent additional inactivation of pathogens due to remaining heat.

2.4. Bacterial enumeration

Treated cherry tomatoes samples were homogenized for 2 min with a mechanical stomacher (EASY MIX, AES Chemunex, Rennes, France) and orange samples were shaken and massaged by hand for 1 min. After homogenization, 1 ml aliquots of samples were 10-fold serially diluted with 9 ml of sterile 0.2% peptone water, and 0.1 ml of appropriate dilutions were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine

Desoxycholate Agar (XLD; Difco), and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate surviving populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. All plates were incubated at 37℃ for 24 h, and then colonies enumerated. To confirm pathogen identity, presumptive colonies were randomly selected from selective media and subjected to biochemical and serological tests. These tests consisted of the *E. coli* O157:H7 latex agglutination assay (Oxoid, Basingstoke, UK), the *Salmonella* latex agglutination assay (Oxoid, Basingstoke, UK), and the API Listeria test (BioMérieux, Hazelwood, MO).

2.5. Color and texture measurement

To determine the effect of SS and SHS treatment on the color of cherry tomatoes and oranges, color assessments were measured using a Minolta colorimeter (Model CR-400; Minolta Camera Co. Ltd., Osaka, Japan). Measurements were taken from SS and SHS treated and untreated samples measured at random locations on cherry tomatoes and oranges and averaged. L*, a*, and b* values indicate lightness, redness, and yellowness of the sample, respectively.

Changes in texture of SS and SHS treated cherry tomatoes and oranges were evaluated with a texture analyzer (TA-CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a cylinder probe. After treated samples were cooled in ice water, a sample was placed onto the press holder, and a 1.5 or 4 mm diameter cylinder blade was moved down at 2

mm/s for evaluating cherry tomatoes and oranges, respectively. Maximum force was recorded using TexturePro CT software (Brookfield Engineering Laboratories, Inc.). Three measurements were performed for each treatment with independently-prepared samples.

2.6 Vitamin C measurement

Vitamin C content in cherry tomatoes and oranges was determined following the method validated by Odriozola-Serrano et al. (2007). Individually, treated cherry tomatoes, orange pulp, and orange peels were homogenized and mixed with 10 ml of solution containing 45 g/L metaphosphoric acid and 7.2 g/L dithiotreitol) in an electric blender (Tefal BL16, France). The resulting mixture was centrifuged at 15,300 g for 15 min at 4℃ and a 10 μL aliquot of the supernatant was injected into a high-performance liquid chromatography (HPLC; Ultimate 3000; Dionex, Sunnyvale, CA) equipped with an autosampler and an UV detector set at 265 nm. A reversed-phase C18 column (5-μm particle size, 4.6-mm diameter, 250-mm length; Dionex) was used to separate the ascorbic acid using 50 mM potassium phosphate buffer (pH 7.2) and acetonitrile (95:5 [vol/vol]) as a mobile phase. The mobile phase was filtered using a 0.45-μm-pore-size membrane filter (Micron Separations, Inc., Westboro, MA) and degassed via vacuum before being applied to the column. A flow rate of 0.5 ml/min was used, and the retention time was 3.7 min. A standard calibration curve was

obtained by using L-ascorbic acid (Sigma Chemical Co., St. Louis, MO) in concentrations ranging from to 5 to 80 mg/100 ml.

2.7. Determination of antioxidant capacity

The antioxidant capacities of cherry tomatoes, orange pulp, and orange peels were assayed through evaluation of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect. This determination was based on the method validated by De Ancos et al. (2002). This assay was carried out employing a reaction mixture of aliquots (0.010 ml) of the sample supernatant in 3.9 ml of methanolic $DPPH^{\cdot}$ (0.025 g/L) and 0.090 ml of distilled water of centrifuged at 6000 g for 15 min at 4℃. The samples were shaken vigorously and kept in darkness for 30 min. The absorption of the samples was measured spectrophotometrically using a (Spectramax M2e; Molecular Devices, Sunnyvale, CA) at 517 nm. Results were expressed as the percentage of inhibition of the radical $DPPH^{\cdot}$, that is, the decrease in absorbance with respect to the control value ($DPPH^{\cdot}$ initial absorption value).

2.8. Statistical analysis

All experiments were repeated three times with duplicate samples. Triplicate data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple-range test at a probability level of $P < 0.05$.

II(2)-3. Results

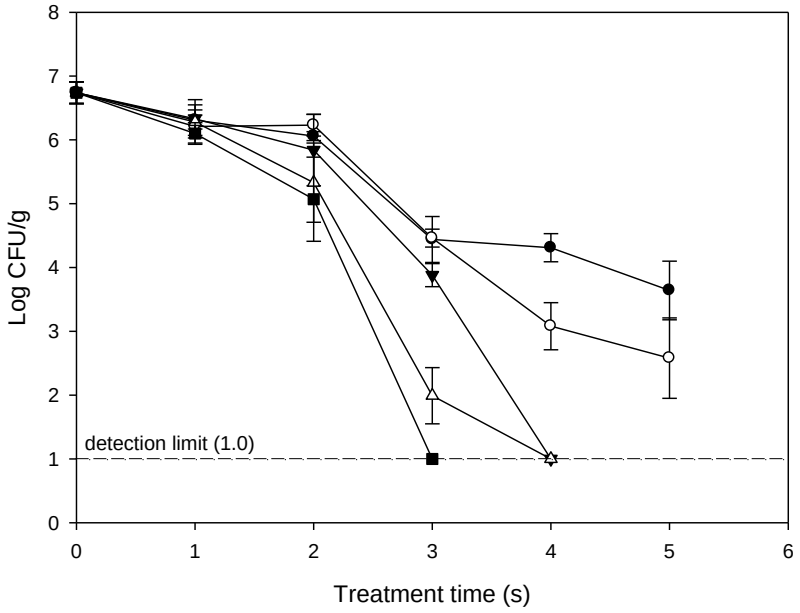
3.1. Inactivation of pathogenic bacteria on cherry tomatoes and oranges

Populations (log CFU/g) of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on cherry tomatoes and oranges following SS and SHS treatment are depicted in Fig. 2 and 3. Initial inoculum levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on cherry tomatoes were 6.74, 7.05, and 6.21 log CFU/g and those on oranges were 6.91, 6.75, and 6.27 log CFU/g, respectively. As the temperature and duration of SHS treatment increased, surviving populations of the three pathogens decreased dramatically. After SHS treatment at 200℃ for 3 s, levels of the three pathogens on cherry tomatoes were reduced to below the detection limit (1 log CFU/g), whereas SS treatment at 100℃ achieved only 2.30, 2.66, and 1.39 log reductions of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Populations of the three pathogens on oranges were greatly reduced to undetectable levels (1.7 log CFU/g) when treated with SHS treatment at 200℃ for 20 s, while *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* experienced log reductions of 2.57, 2.33, and 2.42 after SS treatment at 100℃ for the same time interval. It was observed that SHS treatment caused an additional 3.39–3.82 and 2.15–2.72 log reductions of the three pathogens on cherry tomatoes and oranges, respectively, compared to SS treatments. In addition, populations of the three pathogens on cherry tomatoes were reduced to below the detection limit when subjected to heating for 4 s at

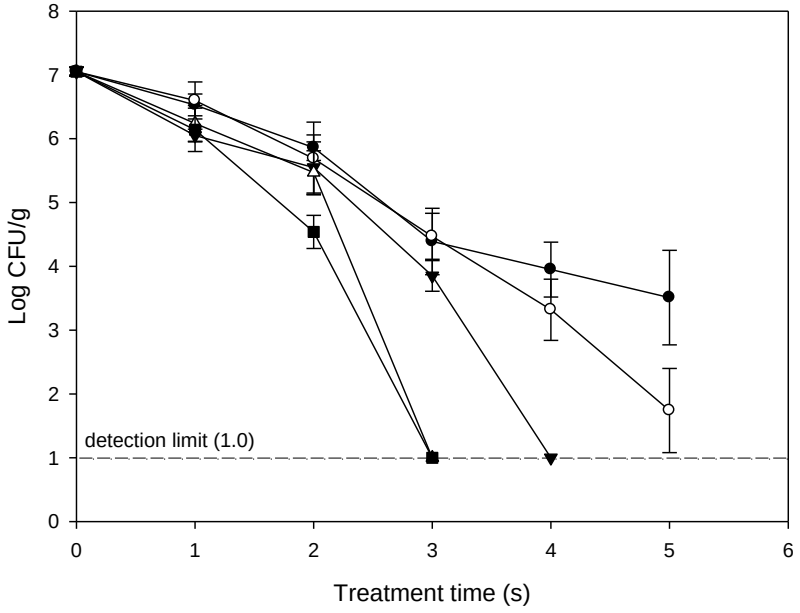
150℃ and at 175℃, 4 s at 150℃ and 3 s at 175℃, and 5 s at 150℃ and 4 s at 175℃ for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes,* respectively. In case of oranges, numbers of the three pathogens were reduced to below the detection limit when treated for 30 s at 175℃, 20 s at 175℃, and 20 s at 175℃ for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes,* respectively.

These results indicate that increasing temperature up to 200℃ for SHS treatment promote the inactivation efficacy on cherry tomatoes and oranges compared to SS treatment.

A


Log CFU/g
Treatment time (s)
detection limit (1.0)
0
1
2
3
4
5
6
7
8

B


Log CFU/g
Treatment time (s)
detection limit (1.0)
0
1
2
3
4
5
6
7
8

C

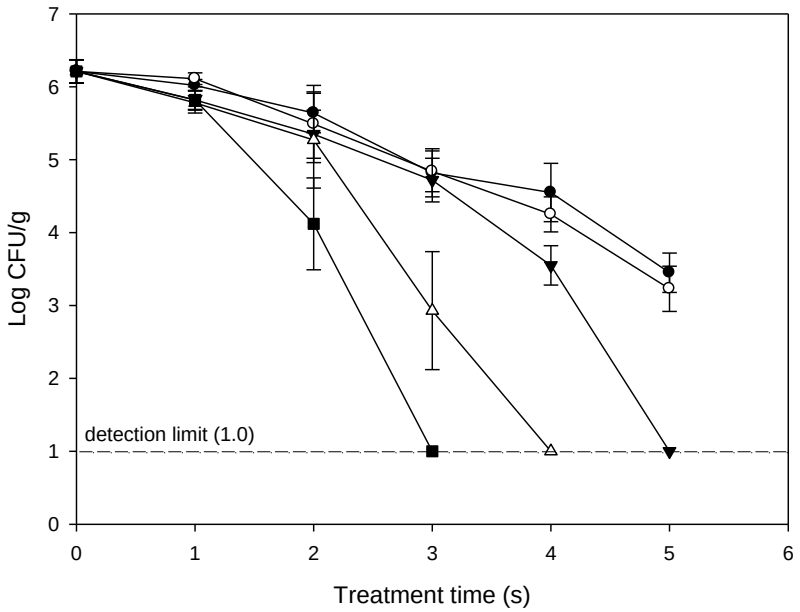
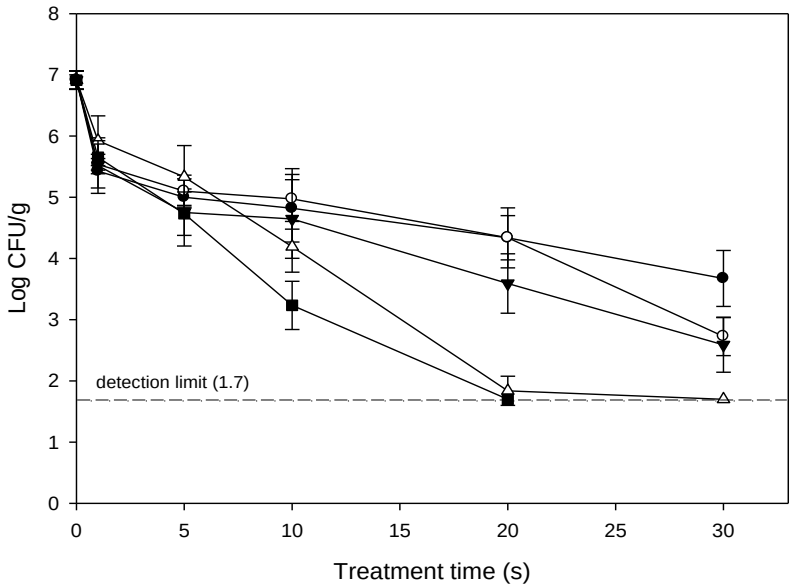
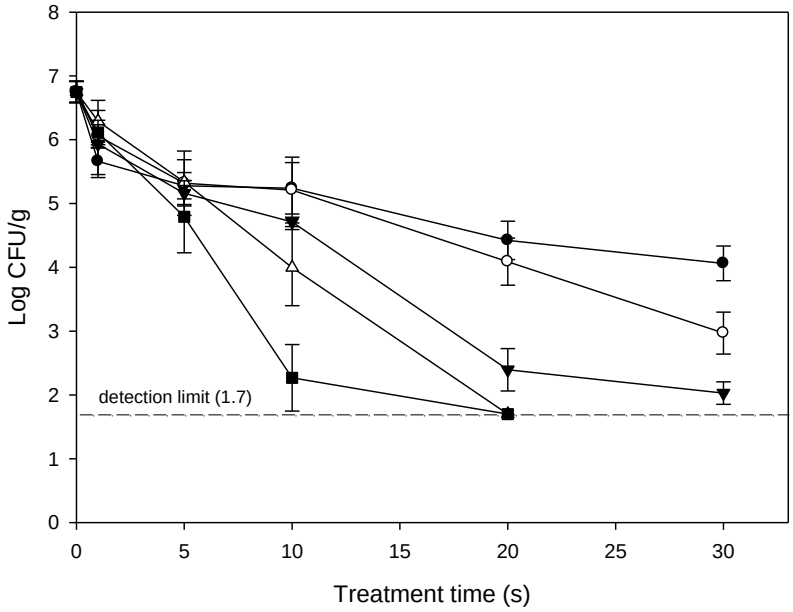


Fig. II(2)-1. Survival curves for *Escherichia coli* O157:H7 (A), *Salmonella* Typhimurium (B), and *Listeria monocytogenes* (C) on cherry tomatoes treated with SS at 100℃ (●), SHS at 125°C (○), SHS at 150°C (▼), SHS at 175°C (△), SHS at 200°C (■).

A


Log CFU/g
detection limit (1.7)
Treatment time (s)

B


Log CFU/g
detection limit (1.7)
Treatment time (s)

C

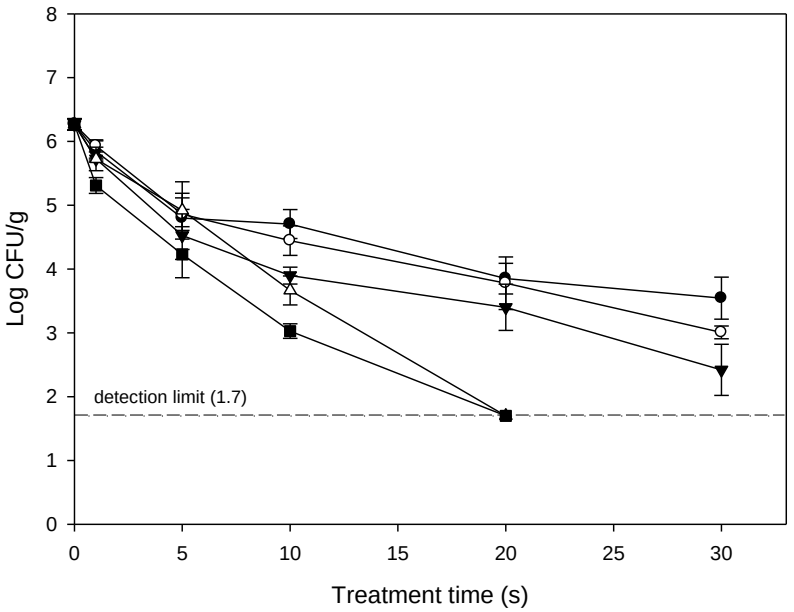


Fig. II(2)-2. Survival curves for *Escherichia coli* O157:H7 (A), *Salmonella* Typhimurium (B), and *Listeria monocytogenes* (C) on oranges treated with SS at 100°C (●), SHS at 125°C (○), SHS at 150°C (▼), SHS at 175°C (△), SHS at 200°C (■).

3.2. Effect of SS and SHS treatment on color and texture of cherry tomatoes and oranges

The color values of cherry tomatoes and oranges after SS and SHS treatment are summarized in Table 1. The color (L*, a*, and b*) values for SS and SHS treated cherry tomatoes and oranges were not significantly ($P > 0.05$) different from those of untreated samples. Although measured three color values of samples were irregular due to natural color variations in fresh produce, statistically significant differences were not observed during the entire treatment interval (Table 1). Also, SS and SHS treatment duration for 5 s and 30 s on cherry tomatoes and oranges, respectively, did not significantly ($P > 0.05$) change the maximum load values of the texture measurements.

Table II(2)-1. Color analysis of steam treated cherry tomatoes (A) and oranges (B) where L* is lightness, a* is redness, and b* is yellowness

(A) Cherry tomatoes

Color Parameter	Temperature (℃)	Treatment time (s) 0	1	2	3	4	5
L*	25	37.97 ± 1.55 A	-	-	-	-	-
	100	-	37.52 ± 0.63 A	37.92 ± 0.91 A	38.03 ± 1.73 A	38.19 ± 0.95 A	37.96 ± 0.80 A
	125	-	37.07 ± 1.30 A	37.61 ± 0.43 A	38.54 ± 1.08 A	38.43 ± 1.19 A	38.32 ± 1.01 A
	150	-	37.39 ± 0.80 A	38.19 ± 0.57 A	37.91 ± 0.66 A	38.74 ± 1.46 A	38.25 ± 1.47 A
	175	-	38.00 ± 0.79 A	38.37 ± 1.68 A	37.70 ± 0.94 A	37.52 ± 1.12 A	38.19 ± 0.41 A
	200	-	38.46 ± 1.11 A	37.47 ± 0.90 A	37.65 ± 0.66 A	38.28 ± 0.42 A	38.10 ± 0.99 A
a*	25	15.82 ± 0.87 A	-	-	-	-	-
	100	-	15.53 ± 0.45 A	15.30 ± 0.49 A	15.32 ± 0.67 A	15.79 ± 0.59 A	14.70 ± 0.73 A
	125	-	15.71 ± 0.78 A	15.58 ± 0.13 A	16.31 ± 0.48 A	16.23 ± 0.92 A	16.01 ± 0.45 A
	150	-	15.83 ± 0.82 A	15.62 ± 0.94 A	16.38 ± 0.81 A	16.16 ± 0.32 A	15.86 ± 0.24 A
	175	-	15.81 ± 0.38 A	15.64 ± 0.51 A	16.33 ± 0.55 A	16.24 ± 0.94 A	16.34 ± 0.34 A
	200	-	15.42 ± 0.99 A	15.53 ± 0.24 A	16.51 ± 0.39 A	16.26 ± 0.89 A	16.13 ± 0.55 A
b*	25	18.00 ± 0.37 A	-	-	-	-	-
	100	-	18.75 ± 0.42 A	18.23 ± 0.41 A	18.04 ± 0.12 A	18.39 ± 0.38 A	18.42 ± 0.43 A
	125	-	17.49 ± 0.72 A	18.18 ± 0.32 A	18.52 ± 0.72 A	18.89 ± 0.66 A	18.73 ± 0.67 A
	150	-	18.35 ± 0.44 A	18.54 ± 0.64 A	18.11 ± 0.13 A	18.93 ± 0.82 A	18.89 ± 0.62 A
	175	-	18.86 ± 0.77 A	18.25 ± 0.21 A	18.31 ± 0.22 A	18.04 ± 0.31 A	18.25 ± 0.29 A
	200	-	18.07 ± 0.26 A	18.18 ± 0.28 A	18.88 ± 0.63 A	18.54 ± 0.22 A	18.41 ± 0.55 A

(B) Oranges

Color Parameter	Temperature (℃)	Treatment time (s) 0	1	5	10	20	30
L*	25	71.75 ± 1.58 A	-	-	-	-	-
	100	-	70.58 ± 1.89 A	71.50 ± 0.63 A	70.51 ± 1.31 A	72.02 ± 1.32 A	71.05 ± 1.59 A
	125	-	71.45 ± 1.13 A	72.49 ± 0.96 A	71.26 ± 1.35 A	72.29 ± 1.24 A	72.11 ± 1.49 A
	150	-	71.79 ± 0.90 A	71.71 ± 1.74 A	71.63 ± 0.55 A	70.59 ± 1.77 A	72.19 ± 1.47 A
	175	-	70.28 ± 1.70 A	71.06 ± 0.64 A	71.75 ± 1.18 A	71.43 ± 0.55 A	70.55 ± 1.07 A
	200	-	72.03 ± 1.40 A	73.08 ± 1.87 A	70.64 ± 1.42 A	71.52 ± 0.68 A	71.99 ± 0.68 A
a*	25	10.70 ± 1.03 A	-	-	-	-	-
	100	-	9.77 ±1.32 A	9.34 ± 1.85 A	9.31 ± 1.86 A	10.42 ± 1.41 A	9.09 ± 2.11 A
	125	-	9.71 ±1.23 A	9.21 ± 1.31 A	9.65 ± 1.43 A	9.82 ± 2.24 A	10.71 ± 0.61 A
	150	-	9.06 ±1.76 A	10.75 ± 1.00 A	10.23 ± 1.11 A	9.68 ± 1.83 A	8.40 ± 2.95 A
	175	-	10.35 ± 1.26 A	10.69 ± 0.89 A	9.80 ± 1.10 A	9.63 ± 1.20 A	9.23 ± 1.55 A
	200	-	9.13 ± 1.75 A	9.33 ± 1.55 A	11.35 ± 1.86 A	9.81 ± 1.84 A	9.41 ± 1.85 A
b*	25	65.72 ± 1.98 A	-	-	-	-	-
	100	-	64.87 ± 1.39 A	65.23 ± 1.20 A	64.59 ± 1.85 A	64.30 ± 1.48 A	66.37 ± 2.04 A
	125	-	66.47 ± 1.41 A	65.55 ± 1.17 A	64.16 ± 2.47 A	65.48 ± 0.57 A	66.69 ± 2.01 A
	150	-	66.75 ± 1.33 A	65.26 ± 1.63 A	64.10 ± 1.81 A	64.78 ± 1.63 A	64.28 ± 2.67 A
	175	-	65.33 ± 0.99 A	66.98 ± 2.34 A	66.00 ± 0.67 A	64.57 ± 1.46 A	64.26 ± 1.87 A
	200	-	66.00 ± 1.26 A	64.36 ± 2.22 A	65.57 ± 1.10 A	65.21 ± 0.63 A	64.81 ± 2.33 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

Table II(2)-2. Maximum load values for texture of cherry tomatoes (A) and oranges (B) following treatment with SS and SHS

(A) Cherry tomatoes

	Treatment time (s)					
Temperature (℃)	0	1	2	3	4	5
25	16.52 ± 1.43 A	-	-	-	-	-
100	-	16.05 ± 1.11 A	16.76 ± 1.18 A	16.43 ± 0.92 A	16.59 ± 0.68 A	16.22 ± 0.92 A
125	-	16.26 ± 1.42 A	16.25 ± 0.42 A	16.43 ± 1.42 A	16.69 ± 0.49 A	16.24 ± 1.32 A
150	-	16.86 ± 1.21 A	16.18 ± 0.82 A	16.78 ± 1.04 A	16.58 ± 1.12 A	16.29 ± 1.04 A
175	-	16.95 ± 1.08 A	16.56 ± 0.22 A	16.55 ± 0.53 A	16.14 ± 0.97 A	16.78 ± 1.23 A
200	-	16.52 ± 0.59 A	16.85 ± 0.84 A	16.43 ± 0.77 A	16.27 ± 0.66 A	16.18 ± 0.95 A

(B) Oranges

	0	1	5	10	20	30
25	37.60 ± 3.44 A	-	-	-	-	-
100	-	35.90 ± 3.90 A	38.82 ± 4.40 A	38.23±3.92 A	38.37 ± 5.35 A	36.92 ± 3.95 A
125	-	40.39 ± 4.11 A	38.00 ± 3.11 A	37.77±3.21 A	38.78 ± 4.20 A	36.88 ± 5.45 A
150	-	39.22 ± 2.51 A	35.97 ± 5.85 A	35.55±4.73 A	40.40 ± 5.93 A	36.19 ± 4.11 A
175	-	35.00 ± 2.17 A	35.68 ± 3.73 A	37.10±4.89 A	36.89 ± 3.71 A	39.20 ± 4.19 A
200	-	40.15 ± 4.30 A	35.05 ± 3.90 A	35.45±3.30 A	37.03 ± 4.37 A	37.49 ± 3.55 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

3.3. Effect of SS and SHS treatment on vitamin C and antioxidant capacities of cherry Tomatoes, orange pulp, and orange peels

The vitamin C content of untreated cherry tomatoes, orange pulp, and orange peels was 21.02 ± 1.84, 48.90 ± 4.23, and 28.29 ± 3.89 mg/100g, respectively. SS and SHS treated cherry tomatoes, orange pulp, and orange peel had a vitamin C content ranging from 18.89 ± 2.42 to 23.17 ± 2.01, 43.79 ± 5.74 to 53.99 ± 4.68, and 21.49 ± 27.47 mg/100g (Table 3). There were no statistically significant differences in vitamin C content between untreated and treated samples ($P > 0.05$) except orange pulp treated with SS and SHS for 30 s.

Antioxidant capacities of cherry tomatoes, orange pulp, and orange peels were measured as free radical-scavenging capacity in a $DPPH^{\cdot}$ model. Fresh untreated cherry tomatoes, orange pulp, and orange peels exhibited 86.6, 89.4, and 78.3% inhibition, respectively (Table 4). Antioxidant capacity of SS and SHS treated cherry tomatoes, orange pulp, and orange peels were 86.6 to 87.7, 76.2 to 79.3, and 92.1 to 93.3% inhibition of $DPPH^{\cdot}$, respectively, with non-significant differences between SS treated and untreated products ($P < 0.05$). However, antioxidant capacity of orange peels treated with SS and SHS for 30 s decreased and those were shown to be significantly different compared to untreated orange peel samples.

Table II(2)-3. Ascorbic acid contents of cherry tomatoes (A), orange pulp (B), and orange peel (C) following treatment with SS and SHS

(A) Cherry tomatoes

	Treatment time (s)					
Temperature (℃)	0	1	2	3	4	5
25	21.02 ± 1.84 A	-	-	-	-	-
100	-	20.21 ± 1.12 A	20.32 ± 1.02 A	19.02 ± 1.77 A	19.03 ± 1.79 A	19.02 ± 1.91 A
125	-	22.32 ± 1.58 A	19.47 ± 1.97 A	21.67 ± 0.73 A	20.29 ± 1.32 A	19.63 ± 1.58 A
150	-	19.89 ± 1.55 A	19.36 ± 1.79 A	20.29 ± 0.83 A	20.74 ± 0.48 A	18.89 ± 2.42 A
175	-	21.39 ± 0.87 A	20.83 ± 0.54 A	23.17 ± 2.01 A	19.34 ± 1.69 A	20.03 ± 1.79 A
200	-	20.32 ± 1.38 A	18.32 ± 2.10 A	19.66 ± 1.64 A	20.21 ± 1.42 A	20.40 ± 1.82 A

(B) Orange pulp

	Treatment time (s)					
Temperature (℃)	0	1	5	10	20	30
25	48.90 ± 4.23 A	-	-	-	-	-
100	-	53.06 ± 5.22 A	48.44 ± 5.62 A	43.79 ± 5.74 A	58.86 ± 3.21 B	53.48 ± 4.23 AB
125	-	49.52 ± 3.83 A	47.34 ± 4.23 A	47.83 ± 3.18 A	45.12 ± 4.23 A	47.67 ± 2.97 A
150	-	48.32 ± 1.83 A	57.41 ± 2.29 B	45.86 ± 4.22 A	50.24 ± 3.22 A	59.00 ± 3.11 B
175	-	47.59 ± 2.11 A	56.73 ± 1.98 B	46.72 ± 2.27 A	48.03 ± 2.98 A	46.81 ± 4.74 A
200	-	45.67 ± 4.49 A	44.94 ± 4.23 A	53.99 ± 4.68 AB	45.99 ± 3.85 A	46.48 ± 3.19 A

(C) Orange peel

	0	1	5	10	20	30
25	28.29 ± 3.89 A	-	-	-	-	-
100	-	25.97 ± 3.39 A	24.02 ± 5.73 A	24.60 ± 2.34 AB	23.80 ± 6.18 A	22.25 ± 1.98 B
125	-	26.82 ± 3.70 A	25.22 ± 3.66 A	24.53 ± 3.44 A	22.08 ± 3.28 AB	26.69 ± 2.19 A
150	-	26.35 ± 2.64 A	25.95 ± 2.74 A	23.60 ± 5.89 A	26.34 ± 1.89 A	21.49 ± 1.15 B
175	-	27.52 ± 1.42 A	24.99 ± 3.88 A	24.79 ± 4.21 A	24.53 ± 4.33 AB	22.47 ± 4.42 A
200	-	26.51 ± 2.41 A	29.87 ± 1.28 A	27.47 ± 2.19 A	23.07 ± 3.28 AB	22.15 ± 2.87 B

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

Table II(2)-4. Effects of superheated steam on antioxidant capacity of cherry tomatoes (A), orange pulp (B), and orange peel (C) following treatment with SS and SHS

(A) Cherry tomatoes

	Treatment time (s)					
Temperature (℃)	0	1	2	3	4	5
25	86.64 ± 4.85 A	-	-	-	-	-
100	-	86.79 ± 3.07 A	88.31 ± 2.10 A	86.73 ± 3.18 A	85.94 ± 4.25 A	87.71 ± 1.77 A
125	-	86.63 ± 2.22 A	87.07 ± 2.94 A	85.68 ± 3.76 A	86.51 ± 3.49 A	87.06 ± 2.21 A
150	-	88.62 ± 2.63 A	86.58 ± 4.32 A	86.52 ± 3.68 A	86.15 ± 4.74 A	85.75 ± 4.91 A
175	-	86.91 ± 3.23 A	85.47 ± 5.58 A	85.98 ± 3.73 A	85.53 ± 4.49 A	86.14 ± 4.36 A
200	-	85.99 ± 4.27 A	86.02 ± 4.83 A	86.09 ± 4.5 A	87.17 ± 2.99 A	85.63 ± 4.92 A

(B) Orange pulp

	Treatment time (s)					
Temperature (℃)	0	1	5	10	20	30
25	89.42 ± 3.19 A	-	-	-	-	-
100	-	88.02 ± 2.91 A	89.95 ± 3.92 A	89.54 ± 2.85 A	88.85 ± 1.11 A	88.81 ± 2.19 A
125	-	89.09 ± 2.22 A	90.12 ± 1.97 A	88.53 ± 2.95 A	88.18 ± 2.84 A	89.95 ± 2.75 A
150	-	89.44 ± 1.94 A	89.40 ± 1.65 A	90.49 ± 1.89 A	89.04 ± 1.81 A	90.27 ± 1.74 A
175	-	88.96 ± 3.39 A	89.28 ± 0.89 A	89.29 ± 2.65 A	89.47 ± 2.82 A	90.52 ± 1.85 A
200	-	88.49 ± 3.29 A	89.58 ± 1.75 A	88.73 ± 1.47 A	89.57 ± 1.75 A	88.42 ± 2.48 A

(C) Orange peel

	0	1	5	10	20	30
25	78.32 ± 4.21 A	-	-	-	-	-
100	-	76.92 ± 2.19 A	79.01 ± 3.11 A	78.24 ± 1.88 A	77.59 ± 2.44 A	78.89 ± 3.62 A
125	-	77.11 ± 3.10 A	78.22 ± 2.68 A	78.33 ± 1.94 A	78.12 ± 1.31 A	77.90 ± 2.19 A
150	-	77.99 ± 3.11 A	77.21 ± 3.19 A	78.32 ± 2.84 A	78.04 ± 2.38 A	79.12 ± 2.65 A
175	-	78.26 ± 4.08 A	77.42 ± 2.08 A	78.92 ± 3.18 A	77.74 ± 2.22 A	79.21 ± 1.95 A
200	-	77.97 ± 1.22 A	78.92 ± 1.01 A	76.97 ± 3.02 A	77.39 ± 1.74 A	77.65 ± 2.18 A

The values are means ± standard deviations from three replications.

Means followed by the same letter in a data series (column) are not significantly different ($P > 0.05$).

II(2)-4. Discussion

Heat treatments have been used as staple means for insect disinfestation, decay control, ripening delay, and maintaining fruit quality during storage (McDonald et al., 1999; Lurie, 1998). Various heating technologies including conventional methods such as hot water, vapor heat, and hot air as well as more advanced methods like far infrared heating and radio frequency heating have been evaluated (Birla et al., 2004; Lurie, 1998; Tanaka et al., 2007). Part of this reason may be due to a growing demand to decrease the use of chemicals against pathogens and insects on fresh produce (Lurie, 1998). Several researchers have observed that heat treatment can be a non-damaging physical substitute for chemical invention. Vicente et al. (2006) observed that strawberries heat-treated in an air oven (45℃, 3 h) had a better general appearance and showed a lower increase in potassium leakage, respiration rate, and pyrogallol peroxide activity and a higher ascorbic acid content, antioxidant capacity, ascorbate peroxidase, and superoxide dismutase activity compare to untreated samples. Hasbullah et al. (2012) determined that mortality of the fruit fly *Bactrocera carambola* reached 100% when exposed to hot water at 43℃ for 30 min or at 46℃ for 15 min. Also, they found that vapor heat-treated star fruit was not significantly affected with regard to fruit weight loss, moisture content, hardness, color, total soluble solids or vitamin C content (Hasbullah et al., 2012). Until now, most studies have only involved moderate heat treatment of fresh produce and there has been no published

research describing inactivation of foodborne pathogens on fresh fruits and vegetables by high temperature treatment. We therefore investigated the effect SS and SHS on inactivation of foodborne pathogens and quality changes of fresh produce occurring during short heating treatment.

Steam at 100℃ has proven to be one of the most effective methods to control bacterial populations on beef and pork carcasses (Trivedi et al., 2007; Trivedi et al., 2008). Trivedi et al (2008) observed that three treatment with commercial household steam cleaners for 30–180 s significantly reduced the population of *L. monocytogenes* (7.61–3.23 log CFU/cm^2), as well as total aerobic (5.68–4.04 log CFU/cm^2) and thermoduric (6.12–2.57 log CFU/cm^2) bacteria on the surface of pork skin ($P < 0.05$). Retzlaff et al. (2004) observed that 98.9℃ steam treatment for 9 s resulted in 3.5 log CFU/cm^2 reductions for *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria innocua*. McCann et al. (2006) observed that *E. coli* O157:H7 numbers declined by 2.53, 3.13, 3.53 and 3.27 (log CFU/cm^2) for beef, chicken meat, chicken skin and pork, respectively, and *S.* Typhimurium DT104 numbers declined by 3.65, 5.23, 6.15 and 2.64 (log CFU/cm^2) for beef, chicken meat, chicken skin and pork, respectively, after 60 s steam treatment. However, long SS treatment times were required for bacterial to be fully inactivated on fresh fruit. In the present study, SS treatment at 100℃ for 3 s only attained 1.4–2.7 log reductions for the three pathogens on cherry tomatoes and SS at 100℃ for 20 s only achieved log reductions of 2.3–2.6 on oranges. Furthermore, peeling of tomato skin occurred when both SS and SHS treatment times exceeded 5 s,

(data not shown). On the other hand, we observed that SHS treatment at 200℃ for 3 s attained full inactivation of the three pathogens on cherry tomatoes without changing the tomato appearance.

When steam condenses on a cooled surface, a continuous film of condensate is formed which creates a thermal resistance to the further flow of heat (Tanner et al., 1968). SHS receives additional heat to raise its temperature above the saturation temperature at a constant pressure and is transformed into low-moisture steam (dry steam) (Cenkowski et al., 2007). When SHS contacts a surface, condensation temporarily occurs and then the condensed water evaporates back into the SHS, since moisture content in the chamber is low (Iyota et al., 2007). On the other hand, surfaces treated with SS experience little or no evaporation due to moisture saturation inside the chamber. The continuous film of condensate can protect bacteria from heat treatment and increase thermal resistance. For this reason, the inactivation effects of SS and SHS treatment on the three pathogens on cherry tomatoes and oranges cannot but differ due to the condensation film resulting from SS treatment.

Although fresh fruits and vegetables are sensitive to heat treatment, high temperature short time SHS treatment at 200℃ for 3 s and 20 s did not influence the quality of cherry tomatoes and oranges, respectively. To date, no research has been published dealing with the effect of high temperature short time SHS heat treatment of fresh fruits and vegetables. Accordingly, quality changes described in this study can be compared with results obtained in other

investigations which dealt with other types of produce or other methods. Roy et al. (2004) observed that total phenolic content and total flavonoid content also increased in steam-processed broccoli. Dewanto et al. (2002) determined that thermal processing elevated total antioxidant activity and bioaccessible lycopene content in tomatoes and produced no significant changes in the total phenolics and total flavonoids content. We found in our previous study, following SHS treatment for up to 20 and 30 s on almonds and pistachios, respectively, color values and maximum load values were not significantly ($P > 0.05$) different from untreated controls (under review).

Up till the present time, many research studies have demonstrated that commonly used conventional sanitizers only have a limited effect in reducing populations of pathogenic bacteria on fruits and vegetables. Lang et al. (2004) observed that populations of *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 on lettuce were reduced by 1.1–1.8 log CFU/lettuce sample when treated with 200 μg/ml chlorine. Lee and Baek (2008) noticed that sodium hypochlorite treatment (100 ppm) for 5 min reduced levels of *E. coli* O157:H7 by 1.1 log CFU/g. Neal et al. (2012) reported that 1 mg/L ozone treatment for 30 min reduced levels of *Salmonella* and *E. coli* O157:H7 on spinach leaves by 1.0 and 0.6 log CFU/g, respectively.

The necessity to develop an alternative technology for sanitation of fresh fruits and vegetables while not concurrently producing quality deterioration has increased. This research demonstrated that SHS treatment leads to effective inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L.*

monocytogenes on cherry tomatoes and oranges, as well as preventing quality deterioration. As interest in thermal treatment of fresh fruits and vegetables has increased, SHS technology has shown itself to be a very promising alternative intervention for improving microbial safety of fruits and vegetables as demonstrated by the authors on cherry tomatoes and oranges, while simultaneously reducing processing time and expense.

Chapter II(3)

A Comparison of Continuous and Intermittent Superheated Steam Treatment for Inactivation of Foodborne Pathogens on Radish Seeds and Alfalfa Seeds

II(3)-1. Introduction

In recent years, consumption of vegetable sprouts has increased rapidly as consumers are becoming increasingly health awareness. These sprouts need no preparation and provide availability and high nutritive value including vitamins, minerals, etc. (Meyerowitz, 1999, Weiss and Hammes, 2003). Seed sprouting provides an excellent environment for the growth of microorganisms including foodborne pathogen due to the aerobic condition, pH, and temperature favorable to mesophiles (Stewart et al., 2001). For more than a decade, accordingly, there has been a growth in the frequency of outbreak linked to the consumption of raw sprouts. In 2009 and 2010, a multistate outbreak of *Salmonella* infection, eventually with 228 and 184 cases, was associated with alfalfa sprouts produced at multiple facilities from seeds that likely originated from a common cultivator, respectively (CDC, 2010; Safranek, 2009). Most outbreaks were related with alfalfa sprouts, however, the largest outbreak were associated with consumption of contaminated radish sprouts and reported approximately 6000 people (Ministry of Health and Welfare of Japan, 1997). The causative bacteria of sprout-related outbreaks were *Escherichia coli* O157:H7 and *Salmonella* spp. (Stewart, 2001a; Stewart, 2001b) and *Listeria monocytogenes* which has been isolated from commercially produced sprouted seeds, but no cases of human listeriosis have been linked to those sprouts (National Advisory Committee on Microbiological Criteria for Foods, 1999).

To inactivate foodborne pathogens on sprout seeds, various methods such as hot water treatment (Bari et al., 2008), chermical treatments (Taormina and Beuchat, 1999a; Weissinger and Beuchat, 2000), gamma irradiation (Thayer et al., 2003), high hydrostatic pressure (Neetoo et al., 2008), ozone (Sharma et al., 2003; Wade et al., 2003), ultrasound (Scouten and Beuchat, 2002) have been evaluated. However, treatment with 20,000 ppm chlorine failed to eliminate the pathogen on seeds containing 2.7 log CFU/g (Taormina and Beuchat, 1999a). Chemical treatment has little antimicrobial effect of less than 2 log CFU/g on seeds (Taormina and Beuchat, 1999a).

Currently, research of superheated steam (SHS) treatment has been evaluated for inactivating of foodborne pathogen on food including chicken skin (Kondjoyan and Portanguen, 2008), almond (Bari et al., 2010), and biofilm on stainless steel and polyvinyl chloride (Ban et al, 2014). SHS is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure (Cenkowski et al., 2007) and has been known as a safe and non-polluting technology with low energy consumption (Chou and Chua, 2001). SHS treatment is able to transfer the large amount of latent heat to food when steam condenses on surface of food due to a low initial temperature of a food surface, which rapidly increases the surface temperature (Iyota et al., 2001, James et al., 2000).

Therefore, the purpose of this study was to compare and evaluate the bactericidal effectiveness of continuous method and intermittent method of SS and SHS on radish seeds and alfalfa seeds, and investigate the germination

rate change following with SS and SHS treatment.

II(3)-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80℃ in 0.7 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37℃ for 24 h, and stored at 4℃. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37℃ for 24 h. Cells of each strain were collected by centrifugation at 4000 × g at 4℃ for 20 min and washed three times with buffered peptone water (BPW; Difco, Sparks, MD). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7-10^8 CFU/ml. Suspended pellets of the three strains of each species were combined to produce a mixed culture cocktail.

2.2. Sample preparation and inoculation

Radish seeds and alfalfa seeds were obtained from Danong Co. Ltd. (Korea), dry seeds (50 g) were individually placed in a sterilized glass beaker (1 liter), and 5 ml of a mixture of *E. coli* O157:H7, *Salmonella* Typhimurium,

and *L. monocytogenes* was inoculated on the seeds in each glass beaker in a laminar flow hood. A sterile plastic spoon was used to mix the seeds with the bacterial suspension for 2 min. After the inoculum was decanted, seeds were placed on a sterile perforated tray lined with four layers of cheesecloth and dried in a biosafety cabinet at room temperature (22 ± 2℃) for 1 to 2 h (Zhao et al., 2010).

2.3. Saturated steam and Superheated steam treatment

SS at 100℃, produced by a SS generator, was introduced into a SHS steam generator through a flexible stainless steel tube. SS was converted into SHS by heating with an electrical resistance heater in the SHS generator. The maximum temperature generated from the SHS generator used in this study was 200℃, which was made by giving additional heat to SS at a 1 atm. During these experiments, the SS and SHS temperature was controlled automatically by means of a temperature sensor and intelligent power module (IPM) in each of the steam generators. After SS and SHS temperature of the inlet into the chamber had been stabilized (following 5 min warm-up time) and then inoculated 5 g radish seeds or alfalfa seeds were placed in a 500 ml glass beaker. A valve placed on top of the treatment chamber was used to control steam flow. Steam passed through the flexible hose and chamber by opening the steam valve. Radish seeds or alfalfa seeds were steam treated for 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 s (continuous treatment) or 1 s steam treatment followed by cooling for 2 min at 25℃ (intermittent treatment), repeatedly. SS

treatment was performed at 100℃ while SHS treatments were performed at 150 and 200℃. The samples were immediately removed from the chamber after each treatment, and they were then put into a stomacher bag with 45 ml of 0.2% peptone water, in ice water to protect additional inactivation of pathogens due to remaining heat (Labplas Inc., Sainte-Julie, Quebec, Canada).

2.4. Bacterial enumeration

Treated radish seeds and alfalfa seeds samples were homogenized for 2 min with a mechanical stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, 1 ml aliquots of samples were serially 10-fold diluted with 9 ml of sterile 0.2% peptone water, and 0.1 ml of appropriate dilutions were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate surviving populations of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. All plates were incubated at 37℃ for 24 h, and then colonies enumerated. To confirm pathogen identity, presumptive colonies were randomly selected from selective media and subjected to biochemical and serological tests. These tests consisted of the *E. coli* O157:H7 latex agglutination assay (Oxoid, Basingstoke, UK), the *Salmonella* latex agglutination assay (Oxoid, Basingstoke, UK), and the API Listeria test (BioMérieux, Hazelwood, MO).

2.5 Determination of seed germination percent.

One hundred seeds were spread evenly on sterile cheesecloth in a 90 mm diameter petri dish. Seeds were germinated at room temperature, and the cheesecloths were moistened daily with water to maintain a high-moisture environment. Seeds were visually examined daily and considered germinated when 2 mm of radical protruded from seed coat. Germination percentage was calculated by averaging total number of seeds germinated in plates at the end of day 4.

2.6. Statistical analysis

All experiments were repeated three times with duplicate samples. Triplicate data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple-range test at a probability level of $P < 0.05$.

II(3)-3. Results

3.1. Inactivation of pathogenic bacteria on radish seeds

Populations (log CFU/g) of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on radish seeds during continuous SS and SHS heating are shown in Table 1. Initial inoculum levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on radish seeds were 6.31, 6.60, and 6.10 log CFU/g. As the temperature and durations of SHS treatment increased, the surviving populations of three pathogens decreased more effectively compared with SS treatment. After continuous SHS treatment at 200℃ for 10 s, three pathogens on radish seeds reduced by 3.56, 3.64, and 3.48 log reductions and, whereas continuous SS treatment at 100℃ achieved 1.51, 2.70, and 2.11 log reductions in *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Populations of the three pathogens on radish seeds were reduced by 2.94, 3.39, and 2.83 when 10 times intermittent SHS treatment at 200℃, while *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* experienced log reductions in three pathogens of 1.33, 1.61, and 1.58, respectively, after intermittent SS treatment at 100℃ for the same treatment (Table 2). It was observed that SHS treatment caused an additional 0.94–2.05 and 0.78–1.61 log reductions in three pathogens of the continuous and intermittent steam treatment, respectively, compared to SS treatments.

Table II(3)-1. Survival of three pathogens on radish seeds treated with continuous saturated steam and superheated steam treatment

E. coli O157:H7	Treatment time (s)										
Temperature (℃)	0	1	2	3	4	5	6	7	8	9	10
100	6.31±0.23Aa	6.18±0.34Aa	5.89±0.18Ba	5.96±0.20ABa	5.91±0.26ABa	5.65±0.12BCa	5.49±0.15Ca	4.96±0.07Da	4.89±0.30Da	4.82±0.55Da	4.80±0.31Da
150	6.31±0.23Aa	6.20±0.21Aa	5.86±0.31ABa	5.80±0.12ABa	5.77±0.29ABa	5.47±0.36Ba	5.20±0.17Ba	4.55±0.45Ca	4.06±0.46CDb	3.99±0.33Db	3.79±0.29Db
200	6.31±0.23Aa	6.07±0.16Aa	5.84±0.28Aa	5.09±0.25Bb	4.67±0.21Bb	4.07±0.13Cb	3.53±0.07Db	3.29±0.28DEb	3.10±0.42DEc	2.90±0.13Ec	2.75±0.31Ec
S. Typhimurium											
	0	1	2	3	4	5	6	7	8	9	10
100	6.60±0.19Aa	6.26±0.29Aba	6.14±0.20Aba	6.05±0.32Ba	5.86±0.23BCa	5.71±0.37BCa	5.43±0.33Ca	4.82±0.15Da	4.54±0.28Da	4.03±0.10Ea	3.90±0.09Ea
150	6.60±0.19Aa	6.06±0.35Aba	5.90±0.17Ba	5.72±0.36Ba	5.59±0.38Ba	5.40±0.39BCa	5.02±0.23Ca	4.59±0.27CDa	4.53±0.23CDa	3.70±0.21Db	3.72±0.15Da
200	6.60±0.19Aa	5.89±0.32ABa	5.47±0.39ABa	5.14±0.16Bb	4.37±0.29Cb	3.74±0.24Db	3.51±0.21DEb	3.30±0.44DEb	3.39±0.23DEb	3.34±0.09Ec	2.96±0.13Fb
L. monocytogenes											
	0	1	2	3	4	5	6	7	8	9	10
100	6.10±0.12Aa	6.06±0.19Aa	6.07±0.28Aa	5.91±0.22Aa	5.82±0.34Aa	5.85±0.22Aa	5.76±0.15Ba	4.63±0.37Ca	4.60±0.45Ca	4.11±0.21CDa	3.99±0.28Da
150	6.10±0.12Aa	6.02±0.22Aa	5.95±0.39Aa	5.81±0.23Aa	5.21±0.23Bab	5.06±0.14Bb	5.02±0.29Bb	4.30±0.49Ca	3.59±0.24Db	3.34±0.08Db	3.24±0.18Db
200	6.10±0.12Aa	5.69±0.42ABa	5.81±0.27ABa	5.40±0.37Ba	4.90±0.32Cb	4.12±0.38Dc	3.80±0.18Dc	3.48±0.20Eb	2.83±0.30Ec	2.89±0.26Ec	2.62±0.36Ec

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

Table II(3)-2. Survival of three pathogens on radish seeds treated with saturated steam and superheated steam for 1 s followed by cooling for 2 min at 25℃

E. coli O157:H7						Number (times)					
Temperature (℃)	0	1	2	3	4	5	6	7	8	9	10
100	6.31±0.23Aa	6.18±0.34Aa	5.94±0.18Aba	5.86±0.19Ba	5.88±0.22Aba	5.59±0.18BCa	5.52±0.16BCa	5.26±0.28Ca	5.21±0.16Ca	5.05±0.32Ca	4.98±0.37Ca
150	6.31±0.23Aa	6.20±0.21Aa	5.75±0.36Aba	5.77±0.22Ba	5.66±0.21Ba	5.33±0.29BCa	5.34±0.37BCa	4.88±0.19Ca	4.66±0.20CDb	4.57±0.22CDa	4.28±0.34Da
200	6.31±0.23Aa	6.07±0.16Aa	5.53±0.22Ba	5.29±0.29BCb	4.71±0.32Cb	4.04±0.24Db	3.72±0.17DEb	3.59±0.09Eb	3.55±0.18Ec	3.47±0.02Eb	3.37±0.21Eb
S. Typhimurium											
	0	1	2	3	4	5	6	7	8	9	10
100	6.60±0.19Aa	6.26±0.29Aa	6.22±0.22Aa	6.08±0.38Aa	5.69±0.13Ba	5.79±0.11Ba	5.60±0.21Ba	5.45±0.38BCa	5.20±0.22BCa	5.13±0.21Ca	4.99±0.47Ca
150	6.60±0.19Aa	6.06±0.35ABa	5.96±0.23Ba	5.54±0.19BCa	5.63±0.18BCa	5.52±0.17Ca	5.39±0.26Ca	4.74±0.22Db	4.62±0.08Db	4.30±0.12Eb	4.06±0.18Eb
200	6.60±0.19Aa	5.89±0.32Ba	5.33±0.28Bb	5.01±0.09Cb	4.42±0.12Db	4.01±0.28Eb	3.72±0.40EFb	3.49±0.14Fc	3.52±0.09EFc	3.50±0.22EFc	3.21±0.28Fc
L. monocytogenes											
	0	1	2	3	4	5	6	7	8	9	10
100	6.10±0.12Aa	6.06±0.19Aa	6.10±0.19Aa	5.81±0.32ABa	5.62±0.22Ba	5.66±0.27Ba	5.16±0.21Ca	4.93±0.49CDa	4.74±0.09Da	4.69±0.27CDa	4.52±0.14Da
150	6.10±0.12Aa	6.02±0.22ABa	5.84±0.11Ba	5.61±0.21BCa	5.28±0.19Cab	5.23±0.29CDa	4.81±0.15Da	4.42±0.22Ea	4.40±0.16Eb	4.12±0.22Eb	4.18±0.26Ea
200	6.10±0.12Aa	5.69±0.42ABa	5.72±0.30ABa	5.50±0.19Ba	4.99±0.21Cb	4.21±0.28Db	3.94±0.16Db	3.76±0.26DEb	3.69±0.05Ec	3.48±0.21EFc	3.27±0.29Fb

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.2. Inactivation of pathogenic bacteria on alfalfa seeds

Populations (log CFU/g) of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on alfalfa seeds during continuous SS and SHS heating are shown in Table 3. Initial inoculum levels of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on alfalfa seeds were 6.35, 6.19, and 5.74 log CFU/g, respectively. After continuous SHS treatment at 200℃ for 10 s, three pathogens on alfalfa seeds reduced by 4.12, 3.9, and 2.99 log reductions and, whereas continuous SS treatment at 100℃ achieved 2.73, 2.68, and 2.22 log reductions in *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, respectively. Populations of the three pathogens on alfalfa seeds were reduced by 3.42, 3.58, and 2.57 when 10 times intermittent SHS treatment at 200℃, while *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* experienced log reductions of 1.65, 1.81, and 1.72, respectively, after SS treatment at 100℃ for the same treatment (Table 4). It was observed that SHS treatment caused an additional 0.79–1.39 and 0.85–1.77 log reductions of the continuous and intermittent steam treatment, respectively, compared to SS treatments.

Table II(3)-3. Survival of three pathogens on alfalfa seeds treated with continuous saturated steam and superheated steam treatment

E. coli O157:H7	Treatment time (s)										
Temperature (℃)	0	1	2	3	4	5	6	7	8	9	10
100	6.35±0.35Aa	5.97±0.12Aa	5.34±0.26Ba	5.35±0.02Ba	5.32±0.12Ba	4.97±0.07Ca	4.38±0.34Da	4.34±0.25Da	4.11±0.38DEa	3.73±0.31Ea	3.62±0.33Ea
150	6.35±0.35Aa	5.70±0.09Ba	5.31±0.03Ca	5.44±0.18Ca	4.97±0.26Da	4.50±0.13Eb	3.99±0.19Fa	3.65±0.27Fb	3.61±0.24Fb	3.35±0.22Ga	3.41±0.17Ga
200	6.35±0.35Aa	5.55±0.11Ba	4.63±0.06Cb	4.70±0.26Cb	4.16±0.34CDb	3.56±0.42Dc	2.68±0.53Eb	2.26±0.24Ec	2.37±0.15Ec	2.32±0.25Eb	2.23±0.14Eb
S. Typhimurium											
	0	1	2	3	4	5	6	7	8	9	10
100	6.19±0.39Aa	5.51±0.46Aba	5.42±0.27Ba	5.41±0.17Ba	4.72±0.09Ca	4.58±0.18Ca	4.51±0.42CDa	4.31±0.56CDa	3.97±0.42CDa	3.57±0.29Da	3.51±0.15Da
150	6.19±0.39Aa	5.40±0.34Ba	5.32±0.35Ba	5.21±0.11Bab	4.53±0.31Cab	4.05±0.45CDa	3.85±0.12Db	3.44±0.17Eb	3.47±0.23Ea	3.33±0.37Ea	3.18±0.16Eb
200	6.19±0.39Aa	5.25±0.33Ba	5.06±0.15Ba	4.71±0.40BCb	4.42±0.11Cb	3.15±0.14Db	2.94±0.56Dec	3.30±0.63Db	2.76±0.29Deb	2.41±0.18Eb	2.29±0.17Ec
L. monocytogenes											
	0	1	2	3	4	5	6	7	8	9	10
100	5.74±0.34Aa	5.37±0.07Aba	4.98±0.32Ba	4.90±0.39Ba	5.04±0.20Ba	5.09±0.28Ba	4.84±0.20Ba	4.05±0.29CDa	4.20±0.15Ca	3.56±0.44Da	3.52±0.08Da
150	5.74±0.34Aa	5.17±0.36ABa	4.84±0.13Ba	4.58±0.27BCa	4.50±0.22BCb	4.43±0.17Cb	4.40±0.11Cb	3.82±0.26Da	3.62±0.28Db	3.42±0.17DEa	3.15±0.18Eb
200	5.74±0.34Aa	5.13±0.10Bb	4.76±0.27BCa	4.23±0.35Ca	3.85±0.43CDc	3.58±0.29Dc	3.10±0.30DEc	3.18±0.42DEb	3.18±0.25DEc	2.60±0.55Eb	2.75±0.20Ec

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

Table II(3)-4. Survival of three pathogens on alfalfa seeds treated with saturated steam and superheated steam for 1 s followed by cooling for 2 min at 25℃

E. coli O157:H7	Number (times)										
Temperature (℃)	0	1	2	3	4	5	6	7	8	9	10
100	6.35±0.35Aa	5.90±0.13Aa	5.76±0.12Ba	5.41±0.18Ca	5.14±0.20CDa	4.81±0.17Da	4.88±0.11Da	4.80±0.45Da	4.77±0.38Da	4.75±0.09Da	4.70±0.27Da
150	6.35±0.35Aa	5.76±0.22Ba	5.57±0.10Ba	5.04±0.19Ca	4.88±0.07Cb	4.34±0.23Db	4.29±0.06Db	4.17±0.23Da	3.79±0.08Eb	3.69±0.26Eb	3.44±0.13Eb
200	6.35±0.35Aa	5.59±0.10Ba	5.27±0.18Cb	4.50±0.39Db	3.98±0.41Dc	3.20±0.18Ec	3.16±0.13Ec	3.09±0.34Eb	2.99±0.28Ec	2.92±0.37Ec	2.93±0.33Ec
S. Typhimurium											
˚C	0	1	2	3	4	5	6	7	8	9	10
100	6.19±0.39Aa	5.70±0.23Aba	5.42±0.20Ba	5.01±0.10Ca	5.01±0.14Ca	4.94±0.39CDa	4.46±0.19CDa	4.57±0.16Da	4.60±0.45CDa	4.42±0.37Da	4.38±0.16Da
150j8	6.19±0.39Aa	5.40±0.34Ba	5.23±0.07BCa	4.89±0.09Ca	4.63±0.29Ca	4.12±0.38CDb	4.01±0.07Db	3.76±0.24DEb	3.44±0.32Eb	3.44±0.13Eb	3.39±0.21Eb
200	6.19±0.39Aa	5.25±0.33Ba	4.82±0.35BCb	4.37±0.33Cb	4.30±0.17Ca	3.39±0.35DEc	3.90±0.31CDb	3.68±0.44CDb	3.10±0.52DEb	2.98±0.21Ec	2.61±0.39Ec
L. monocytogenes											
˚C	0	1	2	3	4	5	6	7	8	9	10
100	5.74±0.34Aa	5.37±0.07A	4.81±0.32B	4.87±0.33B	4.73±0.25B	4.68±0.24B	4.59±0.35BC	4.18±0.19C	4.22±0.19C	4.06±0.07C	4.02±0.39C
150	5.74±0.34Aa	5.15±0.13B	4.58±0.48BC	4.60±0.17C	4.48±0.39CD	4.35±0.24CD	4.14±0.28D	3.87±0.06DE	3.74±0.36DE	3.65±0.17E	3.52±0.23E
200	5.74±0.34Aa	5.13±0.10B	4.76±0.24C	4.28±0.18D	3.99±0.43DE	3.68±0.27E	3.59±0.06EF	3.53±0.47EF	3.59±0.16EF	3.35±0.07EF	3.17±0.40F

Data represent means ± standard deviations.

Means with the same capital letter in the same column are not significantly different ($P < 0.05$).

Means with the same lowercase letter in the same row are not significantly different ($P < 0.05$).

3.3. Effect of SS and SHS treatment on germination rate of radish seeds and alfalfa seeds

The germination rates of radish seeds and alfalfa seeds after SS and SHS treatment are summarized in Table 5 and 6, respectively. Compared to radish seeds and alfalfa seeds treated in distilled water (control), continuous steam treatments significantly ($P < 0.05$) reduced percent germination. A continuous steam treatment for 3 and 2 s resulted in a considerably drop in percent germination compared to the water control for radish seeds and alfalfa seeds, respectively. However, 10 times intermittent SHS treatment at 200℃ did not decrease germination rate under the 90% for radish seeds and alfalfa seeds.

Table II(3)-5. Germination percentage (%) of radish seeds after continuous (A) and intermittent (B) SS (100℃) and SHS (150 and 200℃) treatment

(A)

Tempera-ture (℃)	Treatment time (s) 0	1	2	3	4	5	6	7	8	9	10
100	100	100	99	97	29	33	17	6	3	0	0
150	100	100	97	75	33	33	14	0	0	0	0
200	100	100	95	55	25	5	0	0	0	0	0

(B)

	Number (times) 0	1	2	3	4	5	6	7	8	9	10
100	100	100	100	97	99	99	99	98	97	95	96
150	100	100	100	96	98	99	99	97	96	95	94
200	100	100	100	99	100	99	99	97	95	96	94

Table II(3)-6. Germination percentage (%) of alfalfa seeds after continuous (A) and intermittent (B) SS (100℃) and SHS (150 and 200℃) treatment

(A)

Tempera-ture (℃)	Treatment time (s) 0	1	2	3	4	5	6	7	8	9	10
100	98	97	86	82	74	61	45	31	6	0	0
150	98	98	81	79	62	58	34	28	0	0	0
200	98	97	75	68	51	27	11	0	0	0	0

(B)

	Number (times) 0	1	2	3	4	5	6	7	8	9	10
100	99	99	98	99	99	99	98	98	97	95	93
150	99	98	98	98	98	98	99	99	96	95	92
200	99	98	99	99	99	99	97	98	94	91	90

II(3)-4. Discussion

In 1999, the U.S. Food and Drug Administration (FDA) recommended that seeds for sprouting should be treated with 20,000 ppm calcium hypochlorite (FDA, 1999). This method as an intervention for disinfecting contaminated seeds appears to be effective as proved by a 1999 *Salmonella* outbreak in multistate that was traced to a contaminated a contaminated seed lot obtained from California (Weissinger and Beuchat, 2000). However, treatment of alfalfa seeds using the recommended exposure to 20,000 ppm calcium hypochlorite as the source of hypochlorous acid were reported to produce a significant decrease in populations of the target pathogen, but does not completely eliminate *E. coli* O157:H7 (Taormina and Beuchat, 1999a). Furthermore, the use of 20,000 ppm calcium hypochlorite can result in hazardous fumes and cause skin irritation to workers and the environment (Beuchat, 1998; Weissinger and Beuchat, 2000). Although numerous other chemical sanitzers have been examined as alternative to chlorine to disinfect pathogens on alfalfa seeds (Beuchat, 1997; Taormina and Beuchat, 1999a; Taormina and Beuchat, 1999b), none has been proved to be completely effective. It may be occurred that the pathogens outside of the seeds was disinfected but the remaining contamination spread through the seed interior (Enomoto et al., 2002).

Seeds of alfalfa sprouts have comparatively weak coats and can be broken easily result from harvest by machines (Wick, 1999). If the seeds are

contaminated by pathogen from the outside, the interior of the seeds can be contaminated. Although most chemicals may enter damaged seeds, they are not believed to affect the interior of intact seeds (Enomoto et al., 2002). Consumers prefer not to use high concentration chemicals on sprouted vegetables because of foods which are often eaten fresh or only lightly cooked after short periods of cultivation (about a week) (Enomoto et al., 2002). As consumers are becoming increasingly demand for minimally processed, no chemical, additive-free, shelf-stable foods has elicited to find other physical methods as alternatives to chemical or traditional heat treatments (Neetoo et al., 2008).

Saturated steam as traditional heat treatment has proven to be one of the effective methods to control bacterial populations on beef carcasses and pork skin surfaces (Trivedi et al., 2007; Trivedi et al., 2008). Trivedi et al (2007) observed that treatment with commercial available steam unit (Steam Fast, Top Innovation Inc., Riverside, MO, USA) for 60 s reduced the survival of naturally occurring aerobic bacteria (1.8–2.3 log CFU/cm^2), coliforms (1.1–1.6 log CFU/cm^2), and Enterobacteriaceae (1.3–1.9 log CFU/cm^2) at the end of slaughter. Pipek et al. (2006) observed that 90–95℃ steam treatment for 0.013 s at 4–6 bar resulted in 0.4 and 1.3 log CFU/g reductions for naturally occurring mesophiles and psychrophiles, respectively. McCann et al. (2006) observed that *E. coli* O157:H7 and *S.* Typhimurium DT104 numbers decreased by 2.53–3.53 and 2.64–6.15 log CFU/cm^2 for beef, chicken meat, chicken skin, and pork after 60 s steam treatment. However, long steam

treatment times can be affect germination rate of sprout seeds. In the present study, continuous SS treatment at 100℃ for 10 s only attained 1.51–2.7 and 2.22–2.73 log reductions for the three pathogens on radish seeds and alfalfa seeds, respectively. On the other hand, continuous SHS treatment at 200℃ for 10 s achieved 3.48–3.64 and 2.99–4.12 log reductions for the three pathogens on radish seeds alfalfa seeds, respectively. Although continuous steam treatment were effective for inactivation of foodborne pathogens on radish seeds and alfalfa seeds, intermittent steam treatment method as new technology was required to maintain more than 90% germination rate.

In our studies, we could show that intermittent SHS treatment is an alternative to chemicals used to reduce the populations of pathogens on radish seeds and alfalfa seeds. It was observed that a 3 log reduction is achievable for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on radish seeds and alfalfa seeds with SHS treatment. *Salmonella* attaches to alfalfa seeds better than does *E. coli* O157:H7 (Barak et al., 2002). However, in present study, significant difference was not observed for *Salmonella* on both of them and a population on alfalfa seeds was smaller than that of *E. coli* O157:H7.

The results of this study suggest that 9 and 7 times intermittent SHS treatments may be an efficient way to treat of radish and alfalfa seeds prior to sprouting, respectively. This method is easy to implement and in combination with good manufacturing practices and end product testing, the use of strong chlorine treatment can be avoided.

Chapter III

Analysis of Superheated Steam Treatment

Using Computational Fluid Dynamics

III-1. Introduction

A common food processing operations are designed to extend the shelf life of a product by eliminating undesirable microbial activity. Heating process at a specified temperature and time to inactivate the pathogenic bacteria from the food product usually done using hot water, hot air, and steam to treat the food to a temperature and then maintain at that temperature for a period sufficient to kill the microorganisms. A better understanding of the mechanism of the heating process will lead to better processing performance and energy savings (Ghani et al., 1999). If pathogens on food are exposed to thermal treatment, changes in bacteria will result in a reduction of the survival over time. When vegetative cells such as *Escherichia coli*, *Salmonella* spp., or *Listeria monocytogenes* any elevated temperatures above 60℃ would cause such reductions. The most common reference parameter in the thermal treatment is the decimal reduction time (D-value) which has been utilized to quantify the influence of a raised temperature on bacterial survival and determined the influence of various factors on a thermal process.

Thermal treatment is the most common technology used for food preservation today. Superheated steam (SHS) treatment as thermal processing method is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure (Cenkowski et al., 2007) and has been known as a safe and non-polluting technology with low energy consumption (Chou and Chua, 2001). Chaper II-2 research demonstrated that

SHS treatment leads to effective inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on cherry tomatoes and oranges, as well as preventing quality deterioration. The main advantage of the SHS could produce condensed steam to be able to penetrate the void spaces surrounded by closed air to which hot water could not reach because of hydrophobic surface tension (Morgan et al., 1996). By delivering a higher enthalpy to surfaces of foods, it also could increase the possibility to kill pathogens to compare with the SS (Keyes et al., 1936). However, until a recent date, there are a few studies related to the simulation of steam for inactivating of foodborne pathogens.

Computational fluid dynamics (CFD) have been used since long time ago to different industries including aerospace, automotive and nuclear industries, it is only in recent years that they have been applied to food processing. Advances in speed and memory capacity of computers are allowing ever more accurate and rapid calculations to be performed, and lots of commercial software packages of practical utility to the food processing have now be available including COMSOL used in this simulations. CFD can be utilized for predicting mixing efficiency in specific mixer geometry, determining average residence times of turbulent flows through heat exchangers, predicting convection patterns in chillers or ovens, or determining the flow patterns of airborne microorganisms in a clean-room factory environment.

Accurate prediction and analysis of SHS dynamics in treatment chamber based on an understanding of biochemistry and fluid mechanics are very

useful and important in predicting how real process equipment is likely to respond. Chemical and biochemical reactions in food during thermal processing are temperature dependent. These reactions not only eliminate bacteria but also destroy some of the nutrition value. Heat distribution results in unavoidable spatial distributions of the reaction products because such bacterial inactivation processes are all temperature dependents. In this case, bacterial concentration profiles are influenced strongly by the SHS temperature and distribution.

A computational procedure was also developed for describing the changes in live bacteria concentration and its transient spatial distributions during SHS treatment of orange surfaces. SHS at 200℃ was the heating media and orange was used as the model food, having constant properties. The governing equations of continuity were solved together with that of the concentration, using the finite element method and CFD. Arrhenius equation was used to describe the kinetics of bacteria inactivation and the influence of temperature on the reaction rate constant. It was introduced to the software package using COMSOL multi-physics. The objective of this research was to validate SHS heat distribution, flow pattern, and bacterial inactivation in treatment chamber using CFD. This study tried to construct time dependent multi-physics model including compressible fluid dynamics for SHS, heat transfer for the steam and sample food, and chemical reaction for pathogen bacteria based on finite element method (FEM) using irregular triangle mesh to reduce down the computational time.

III-2. Mathematical Model and Simulation

2.1. System configuration

2.1.1 Superheated steam treatment system design

The experimental apparatus consisted of saturated steam generator with a maximum power of 5 kW at a 220 V input, superheated steam generator with a maximum power of 6 kW at a 220 V input and maximum temperature of 200℃, insulated sample treatment stainless chamber (external diameter 23 cm; external height, 32 cm; internal diameter, 17 cm; internal height, 22.5 cm), and flexible stainless steel connection hose. An inlet and outlet with a diameter of 0.9 cm and 0.2 cm were installed at the middle-top and bottom-wall portions, respectively. To minimize the disturbance of the steam flow, a rectangular-grid type of mesh table with a grid (9 by 9 by 10 cm, mesh distance of 2 cm) was installed at a height of 7 cm from the bottom of the chamber to support samples. To discharge the condensate made from superheated steam in the chamber wall, outlet was installed at the bottom, and the whole system is illustrated as shown in Fig. 1.

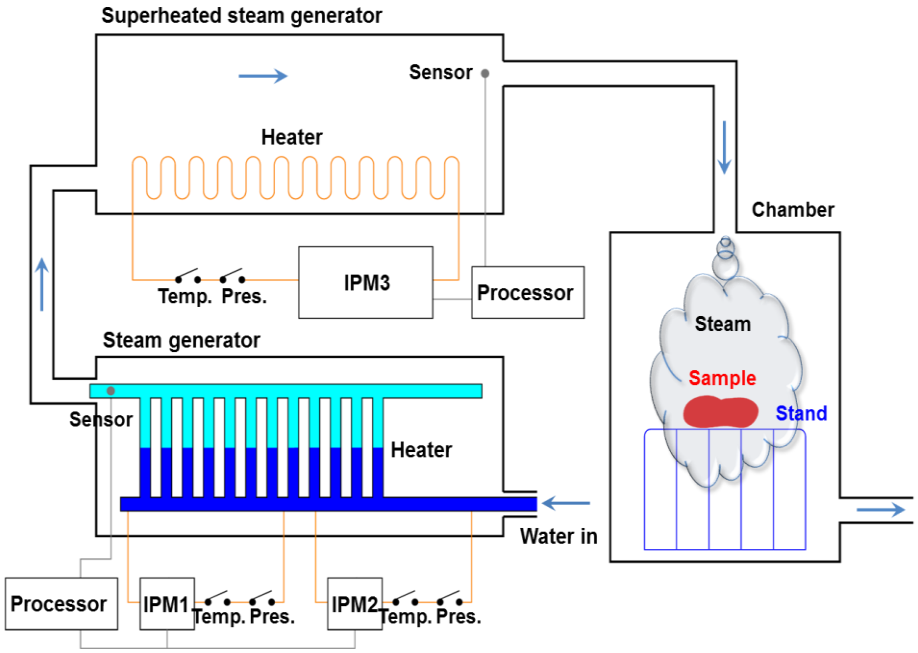


Fig. III-1. Schematic diagram of the superheated steam treatment system.

2.1.2. Temperature monitoring

A fiber optic temperature sensor (FOT-L, FISO Technologies 166 Inc., Quebec, Canada) connected to a signal conditioner (TMI-4, FISO Technologies Inc., 167 Quebec, Canada) was used to measure real-time temperatures during SHS treatment 200℃. Four sensors were installed inner walls of the chamber and the temperature was manually recorded every 1 s. The average values of temperatures were compared with simulation results.

2.2. Mathematical model and simulation

First order chemical reaction, Poisson's, and Navier-Stokes equations were integrated into FEM codes to solve the death of pathogen, heat transfer, and compressible laminar flow, respectively. To prevent excessive mesh distortion induced by longitudinal and circular skin domain of sample, free triangular meshes were manually created by using advancing front method technique for transient state simulation.

2.2.1 Governing equation

The governing equation to consider the diffusion of specie was defined as follow (Bird et al., 2006):

$$\frac{\partial c}{\partial t} + \nabla \cdot (-D\nabla c) + u \cdot \nabla c = R \quad (1)$$

where D is the diffusion coefficient (m^2/s), c is the concentration of specie (%) and R is the reaction rate ($mol/m^3 \cdot s$).

However, in this study, the convection and diffusion related to death of pathogen were not considered. There were no mass flows (fluxes) on the wall as the boundary condition as follow:

$$-n\cdot(-D\nabla c)=0 \qquad (2)$$

The temperature distributions of solid and fluid were obtained by solving the heat transfer equation (Geedipalli *et al.*, 2008; Sabliov *et al.*, 2007):

$$\rho C_p\frac{\partial T}{\partial t}=\nabla\cdot(k\nabla T)-\rho C_p u\nabla T \qquad (3)$$

where T is the temperature (K), t is the time (s), u is the velocity (m/s), ρ is the density (kg/m^3), k is the thermal conductivity (W/m·K), and C_p is the specific heat (J/kg·K). In the heat transfer equation for solid sample, the term $\rho C_p u\nabla T$ was vanished.

Boundary condition with thermal insulation at the wall of chamber was given by:

$$-n\cdot(-k\nabla T)=0 \qquad (4)$$

Because the flow condition and inlet velocity were at compressible state and under Mach 0.3, respectively, Navier-Stokes equation can describe the flow with respect to time and space. The continuum and moment equations as Cartesian coordinates system can be defined as follows:

Continuity equation: $\frac{\partial\rho}{\partial t}+\nabla\cdot(\rho u)=0$ (5)

Navier-Stokes equation: $$\rho\frac{\partial u}{\partial t}+\rho(u\cdot\nabla)u=\nabla\cdot\left[\sigma-\frac{2}{3}\mu(\nabla\cdot u)I\right] \quad (6)$$

where t is the time (s), u is the velocity vector (m/s), σ is the total stress tensor (N/m^2), μ is the dynamic viscosity (pa·s), and I is the identity tensor. The total stress tensor can be expressed as follow:

$$\sigma=-pI+\mu\left[\nabla u+(\nabla u)^{\mathrm{T}}\right] \quad (7)$$

where p is the pressure (Pa), I is the identity tensor, and superscript T is the transpose of a matrix.

Since the fluid is superheated vapor steam and its velocity is very fast, gravitational and buoyancy effects were ignored and then, the Dirichlet boundary condition was given at the walls of chamber as follow:

$$u=0 \quad \text{(No slip)} \quad (8)$$

2.2.2. Prediction of thermo-physical properties

The material properties of steam were imported from embedded COMSOL library (Wagner and Kretzschmar, International Steam Tables, Springer, 2008) and the variables were changed depending on the temperature. The line heat source probe method can be employed for the determination of thermal conductivity and thermal diffusivity simultaneously (Choi and Okos, 1986). In particular, Choi and Okos (1986) proposed, based on literature data, the following general model to predict the density (ρ), specific heat (C_p), thermal conductivity (k) and thermal diffusivity (α) of foods for temperatures.

$$\rho = \sum \rho_i X_i^w \quad (9)$$

$$C_p = \sum C_{pi} X_i^w \quad (10)$$

$$k = \sum k_i X_i^v \quad (11)$$

$$\alpha = \frac{k}{\rho C_p} = \sum \alpha_i X_i^v \quad (12)$$

in which the subscript *i* refers to a particular pure component, estimated volume fraction, $X_i^v = (X_i^w/\rho_i)/\sum(X_i^w \rho_i)$, based on weight fraction X_i^w. Thermal conductivity *k* (W/m°C), thermal diffusivity α (m^2/s) as a function of composition and temperature using given constants.

2.2.3. Bacterial deactivation kinetics

All food processes would cause changes in product quality attributes. These changes may include positive impacts such as the reduction of microbial populations (i.e., bacteria deactivation). It may also include a negative impact on the nutrient concentration and taste. The rate of bacteria deactivation is usually considered to be of the first order kinetics (Reuter, 1993). The reaction rate constant (k_T) is function of temperature and is usually described by Arrhenius equation:

$$k_T = A e^{-E/RT}, \quad (13)$$

where *A* is the reaction frequency factor [s^{-1}], *t* the exposure time [s], *E* the activation energy [kJ $(kg\ mol)^{-1}$], *R* the universal gas constant [kJ $(kg\ mol)^{-1}$ K^{-1}], and *T* is the temperature [°C].

In food process engineering context, the decimal reduction time (D-value)

is often utilized to describe the same relationship. The relationship between the reaction rate constant and the decimal reduction time is (Heldman and Hartel, 1997):

$$k_T = \frac{2.303}{D\text{-value}} \quad . \tag{14}$$

The activation energy used in our simulation is 30 × 104 J mol^{-1}, as reported by Reuter (1993) for distracting microorganisms. The reaction rate constant k_T is then calculated using Eq. (14). Eq. (13) is used to calculate the constant of Arrhenius equation A, giving $A = 2.5 \times 10^{11}$ s^{-1}. The concentration is taken as a dimensionless percentage bacteria concentration defined as the ratio of the true value to the initial concentration of bacteria (multiplied by 100).

2.2.4. Simulation procedure

Numerical simulation of superheated steam was executed by finite element codes based on two-dimensional Cartesian coordinate using COMSOL Multiphysics software (COMSOL 4.3, COMSOL, Inc., Palo Alto, CA). The method analyzed fluid dynamics of steam, heat transfer from steam to sample, and chemical reaction for mortality rates of pathogen. The partial differential equations to govern the different physics were solved simultaneously using the transient mode with a linear solver. Whole domain was divided into four parts: fluid dynamics region, bacteria inactivation boundary (BIB) layer, and peel and pulp parts of sample. The thickness (1

mm) of BIB layer was decided by calculating the averaged corrugation depth of skin part of sample. The gravity term to be able to affect the steam was neglected because the device produce superheated steam and its velocity was fast.

To reduce the computational time and increase the convergence rate, a generalized minimal residual (GMRES), multi-frontal massively parallel sparse (MUMPS), and parallel direct sparse solver (PARDISO) solvers were used for fluid dynamics, heat transfer, and chemical reaction, respectively. Transient analysis with time step of 0.1 s was executed and the Jacobian matrices were updated once per time step.

To increase the mesh quality and minimize the number of mesh, the domains were discretized by free triangle meshes using advanced front method (AFM). The averaged mesh quality (Q) defined as follow (Bank, 1990) was kept to be 0.9637 through whole domain.

$$Q = \frac{4\sqrt{3}A}{h_1^{\,2} + h_2^{\,2} + h_3^{\,2}} \tag{15}$$

where A is the area of triangle, and h_1, h_2 and h_3 are side lengths of triangle.

The mesh sizes for each subdomain were manually controlled and the total numbers of 9,929 triangular meshes were generated. It took 48 hours to finish the simulation using a sever level personal computer (Intel® Xeon® CPU X5690@4.00GHz (2 Processors), G.SKILL RAM 48GB@1,600MHz).

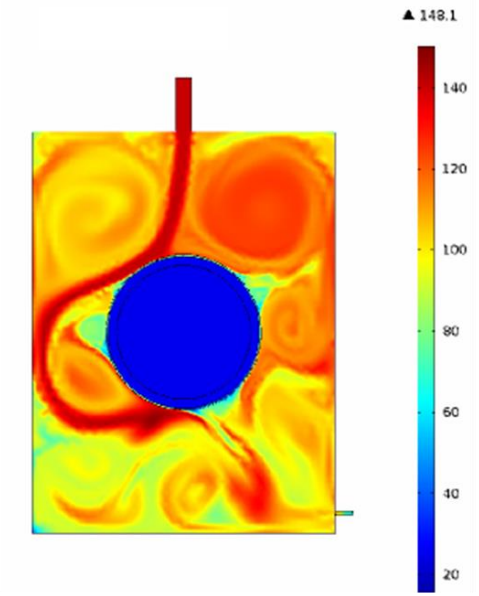
III. Results and Discussion

Fig. 2 represents the results of the simulation for insulated chamber and bacterial populations on orange surface, SHS heated from top of the chamber. The temperature distribution, presented in the form of isotherms, at the early stage of heating (t =1 s) is almost identical to orange SHS treatment (Fig. 2a-1). Fig. 2a-3 shows that during the early stage of heating, the bacteria were killed only at locations close to the top of the steam inlet, and is not influenced by the flow pattern shown Fig. 2a-2.

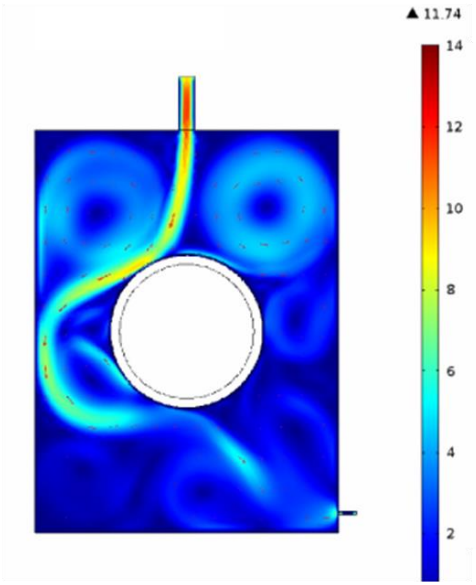
Because the velocity of SHS was fast for the chamber size, the main stream of fluid reached stable state in few seconds. Therefore, compressible flow of SHS was setup for fluid dynamics. The convection and diffusion related to death rate of pathogen was not considered since the total heating time was only 30 s and the sterilization was done on the surface of sample. The simulation introduced new concept named as the BIB layer for the calculation of death rate of bacteria. To validate the code, the temperatures of the thermocouples installed inside the chamber were compared with the simulation results. In the simulation, the fluctuations of temperatures of each location were larger than the experiment results, whereas the average temperatures of simulation were higher about around 20℃ (data not shown). The simulated average outlet velocity of steam had a good agreement with experimental results. The errors occurred in temperatures and outlet velocity as follows; the simulation did not consider energy losses which might be

caused by the contacts between steams and surfaces of chamber, and phase change from vapor to water observed in experiment.

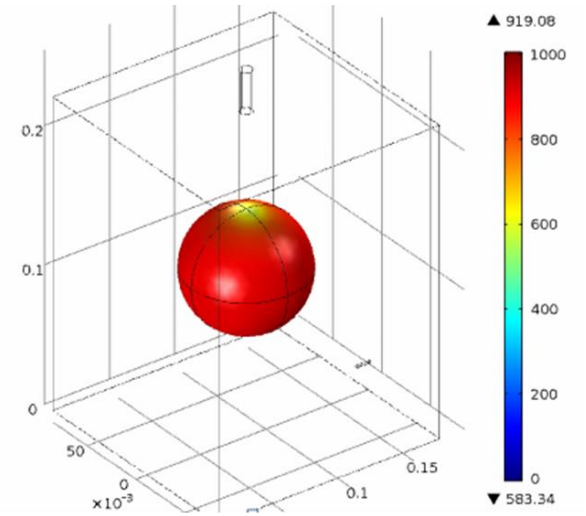
As the bacteria adjacent to the top of the orange, they receive strong heat from the steam inlet. Bacteria away from the top of the orange stays at the initial relative concentration. The isotherms are strongly influenced by turbulent flow of SHS. After 5 s of heating, the bacteria concentration profile shown in Fig. 2b-3 seems to be influenced mainly by the temperature profile (Fig. 2b-1). This is evident from the higher rate of bacteria deactivation at the top of the orange where the temperature is higher. However, the bottom and left of the orange remains little affected with regard to the bacteria death.



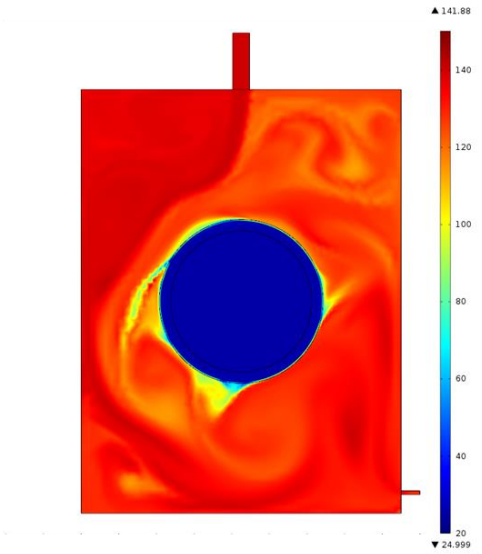
(a-1) Temperature



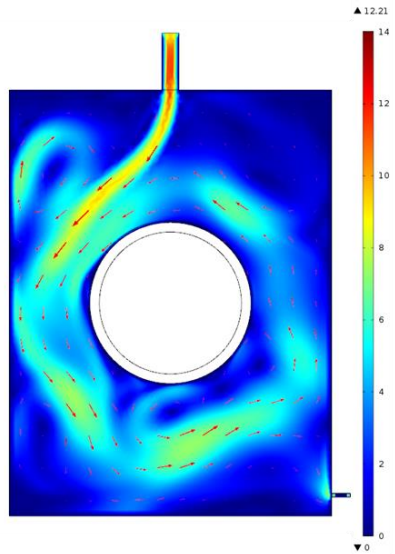
(a-2) Flow pattern



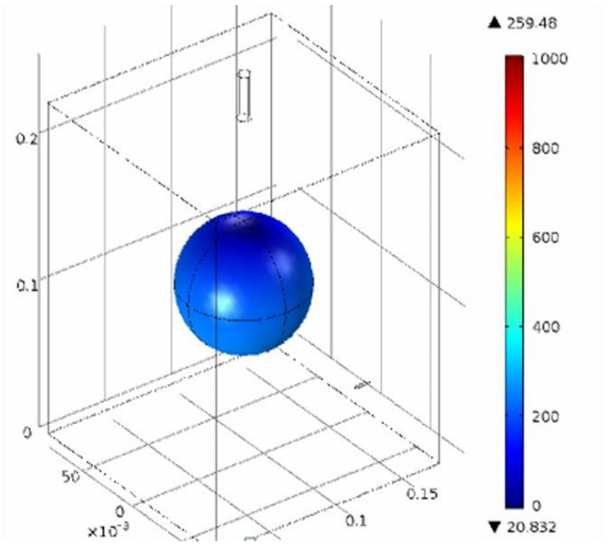
(a-3) Bacteria relative concentration



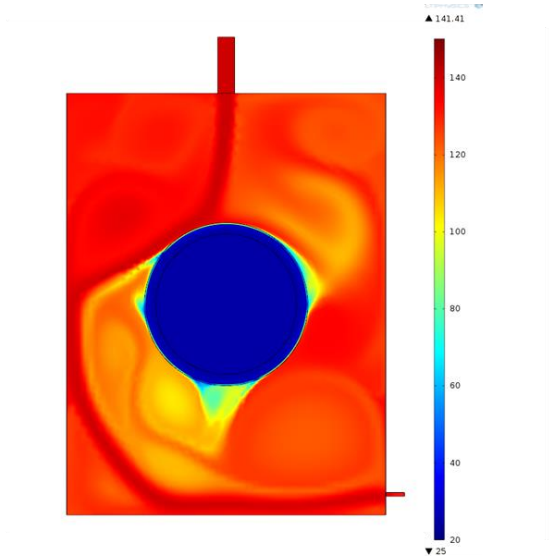
(b-1) Temperature



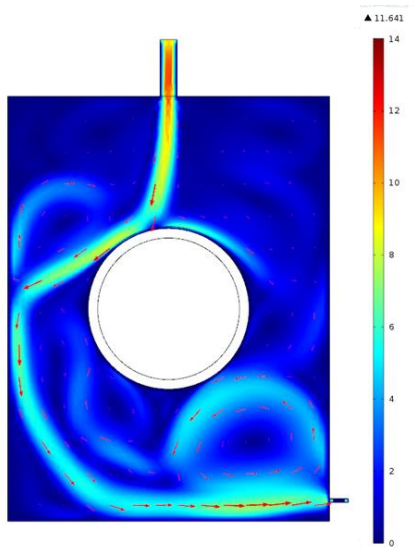
(b-2) Flow pattern



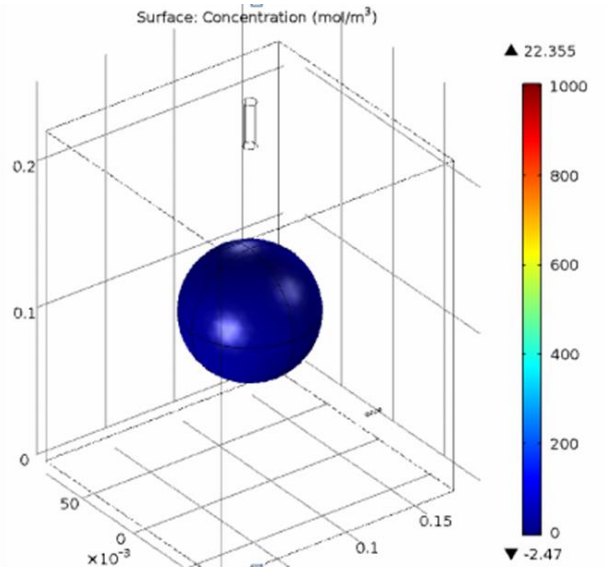
(b-3) Bacteria relative concentration



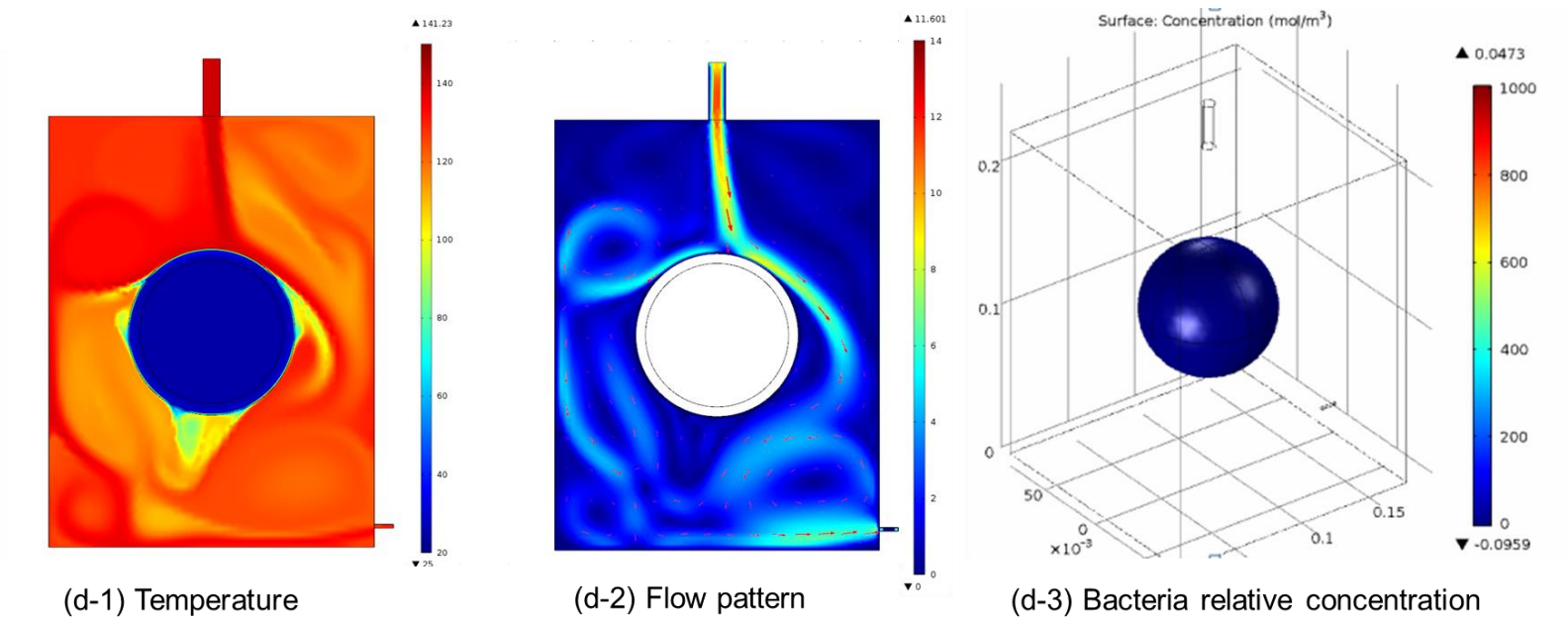
(c-1) Temperature



(c-2) Flow pattern



(c-3) Bacteria relative concentration



(d-1) Temperature (d-2) Flow pattern (d-3) Bacteria relative concentration

Fig. III-2. Temperature, Flow pattern, and bacteria inactivation on an orange in a chamber superheated steam treatment 200℃ for 1 s (a), 5 s (b), 10s (c), and 20 s (d).

Fig. 2 shows the results of the simulation after periods of 10 and 20 s, respectively. The temperature and bacteria concentration profiles are very different from those observed at the beginning of the heating. The appearance of a distinct slowest heating zone (location of the lowest temperature at a given time) shown in Fig. 2c-1 and 2d-1. The orange surface and thus the bacteria carried with it at these locations are exposed to much less thermal treatment. Fig. 2 shows that the bacteria inactivation was influenced significantly by both the temperature and the flow pattern. The highest concentration of bacteria shown in Fig. 2a-1 occurs at two locations. These locations belong to minimum SHS flow velocity and low temperature zones. It is after 20 s of heating that most of the bacteria have been inactivated. Much shorter time would be required for sterilization of orange of fresh produce. In the simulations presented here, the bacteria are assumed to stay with the surface.

E. coli O157:H7 as a sample to test mortality rate of pathogen was selected and the mortalities of the pathogens at different locations were compared with the experiment result averaged on all surfaces of the sample. Because the top part of sample is close to the SHS inlet to chamber, the death ratio of the pathogens was dramatically reduced down to compare with other areas. Because the outlet is located right side of chamber, the possibility that the main stream of steam can occurred in the right side of chamber is over 50%. The phenomenon can increase the temperature of right side of sample.

For this reason, the death ratio at right side was increased to compare with left side.

Fig. 3 compares predicted bacterial inactivation patterns with experimental measurements after treatment with superheated steam at 200℃ The predicted bacterial inactivation values were in good agreement with the experimental data (R^2, 0.95). The prediction error for bacterial inactivation was lower than experimental measurements. These patterns are consistent with the study of Salengke and Sastry (2007), in that enhanced experimental measurements for particle heating was more pronounced than the predicted bacterial inactivation within heated liquid phase and potato particle.

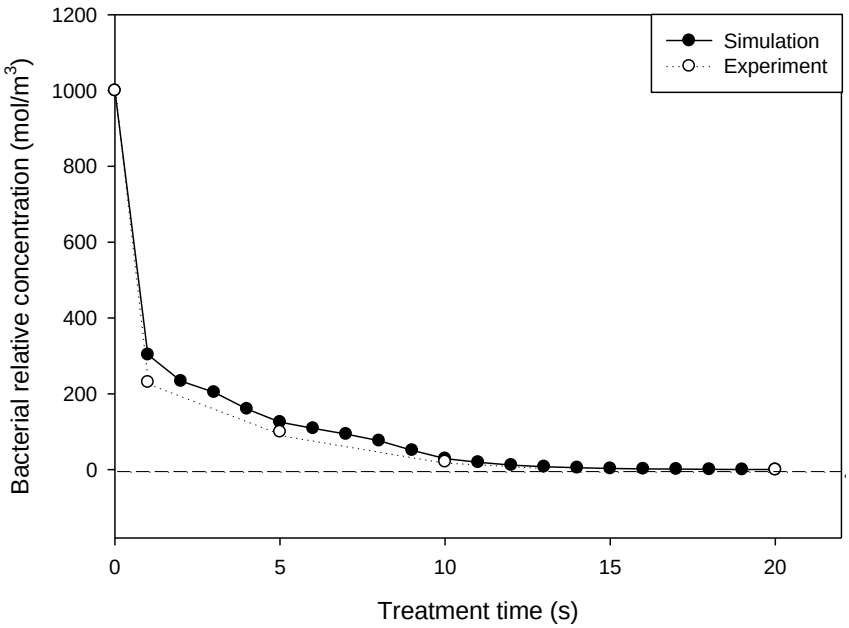


Fig. III-3. Comparison of predicted bacterial inactivation patterns with experimental measurements after treatment with superheated steam at 200℃.

Nomenclature

A	reaction frequency factor [s^{-1}]	p	pressure [Pa]
C	concentration of the bacteria on food sample [number of bacteria m^{-3}]	R	gas constant [kJ $(kg\ mol)^{-1}$ K^{-1}]
C_0	initial concentration of bacteria [bacteria m^{-3}]	t	heating time [s]
C_P	specific heat [J kg^{-1} K^{-1}]	T	temperature [°C]
D	diffusion coefficient [m^2 s^{-1}]	v	velocity in radial direction [m s^{-1}]
E	activation energy [kJ $(kg\ mol)^{-1}$]	α	thermal diffusivity [m^2 s^{-1}]
k	thermal conductivity [W m^{-1} K^{-1}]	μ	apparent viscosity [Pa s]
k_T	reaction rate constant at temperature T [s^{-1}]	ρ	density [kg m^{-3}]

Chapter IV

Development of Portable Superheated Steam Generator and Inactivation Kinetics of Foodborne Pathogens Biofilm Cells

IV-1. Introduction

Biofilms are structured bacterial communities enclosed with polymeric matrices of DNA, protein, and polysaccharides (Stoodley et al., 2002; Sutherland, 2001;Whitchurch et al., 2002), and protect bacterial cells against environmental stresses, detergents, antibiotics, and the host immune system (Bower and Daeschel, 1999; Costerton et al., 1999; Mah and O'Toole, 2001; Yasuda et al., 1994). Accordingly, in terms of food hygiene, biofilms of foodborne pathogens are crucial problems in food processing environments. They may form on a wide variety of abiological surfaces, including stainless steel, polyvinyl chloride, glass, and rubber, which are common materials used in food processing machinery (Prouty and Gunn, 2003; Ronner and Wong, 1993; Ryu et al., 2004), and lead to potential hygiene problems by concomitant bacterial transmission to food products (Shi and Zhu, 2009).

A lot of approaches have been carried out to inactivate biofilms, since conventional methods of controlling planktonic bacteria, including chemical detergents and physical treatments, often prove ineffective. Recently, we demonstrated that superheated steam (SHS) could be utilized to inactivate biofilms of foodborne pathogens on stainless steel and polyvinyl chloride (PVC) surfaces (Ban et al., 2014). SHS is steam which is given additional heat to raise its temperature above the saturation temperature at a constant pressure, and hence, transfers a larger amount of heat to the subject of treatment than saturated steam (James et al., 2000; Topin and Tadrist, 1997). Moreover, the

SHS process is an energy saving and environmentally friendly technology (Tang and Cenkowski, 2000). With its multiple advantages, SHS has been exploited in the disinfection and sterilization processes for food production (Bari et al., 2010; Phungamngoen et al., 2011).

The bacterial inactivation by heat processing technology has been traditionally assumed to follow first-order kinetics. This model assumes that all cells or spores in a population have equal resistance to lethal treatment and therefore a linear relationship between the decline in the logarithm of the number of survivors over treatment time would be expected (Schaffner and Labuza, 1997). D-values are usually determined by calculating the negative inverse of the best-fit line using least-squares linear regression procedure (Linton et al., 1995). However, there has been growing evidence that microbial survival curves may not always linear (Peleg and Cole, 2000) and three kinds of deviations have been observed: curves with a shoulder, curves with tailing, and sigmoidal curves. In consequence various models have been proposed to describe these nonlinear survival curves, such as the Cerf (Cerf, 1977), Weibull (Peleg and Cole, 1998), modified Gompertz (Bhaduri et al., 1991), log-logistic (Cole et al., 1993), Baranyi (Baranyi and Roberts, 1994), Chiruta (Geeraerd et al., 2000), and the Buchanan, Kamau, Whiting-Buchanan, and Xiong (Xiong et al., 1999) models.

The Weibull model has been considered successfully in describing the nonlinear inactivation of different bacteria under various experimental conditions (Buzrul and Alpas, 2004). It has been successfully used to model

thermal inactivation of L. monocytogenes, S. Enteritidis, S. Typhimurium, E. coli O157:H7, and Staphylococcus aureus (Buzrul and Alpas, 2007). The authors pointed out that the estimates obtained using the Weibull model were better than those estimates obtained using first-order kineteics model.

Therefore, the purpose of this study was to model the survival curves of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* biofilm cells on the surfaces of stainless steel by saturated steam and superheated steam generated from portable superheated steam generator using the linear and Weibull models and compare the goodness-of-fit of these models.

IV-2. Materials and Methods

2.1. Bacterial strains and Culture preparation

Three strains each of *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *S.* Typhimurium (ATCC 19585, ATCC 43971, DT 104), and *L. monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were obtained from the bacterial culture collection at Seoul National University (Seoul, Korea) and used in this study. Stock cultures were stored at −80℃ in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 15% glycerol. Working cultures were streaked onto tryptic soy agar (TSA; Difco), incubated at 37℃ for 24 h, and stored at 4℃. Each strain of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* was grown in 10 ml of TSB at 37℃ for 24 h. Cells of each strain were collected by centrifugation at 5000 g at 4℃ for 15 min and washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The final pellets were resuspended in sterile PBS, corresponding to approximately 10^7–10^8 colony-forming units (CFU)/ml.

2.2. Biofilm formation

Each prepared stainless steel coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing suspensions (30 ml) of either *E. coli* O157:H7, *S.* Typhimurium, or *L. monocytogenes* in PBS (ca. 10^7–10^8 CFU/ml). Coupons in bacterial cell

suspensions (10^7–10^8 CFU/ml) were incubated at 4℃ for 24 h to facilitate the attachment of cells and the coupons were then removed with sterile forceps, immersed in 300 ml of sterile distilled water (22 ± 2℃) and gently stirred for 5 s. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of TSB, and then incubated at 25℃ for 6 days. This method was adapted from Kim et al. (2006).

2.3. Saturated steam (SS) and superheated steam (SHS) treatment

SS at 100℃, produced by a SS generator, was introduced into a SHS steam generator through a flexible tube. SS was converted into SHS by heating with a sheath heater in the SHS generator. The maximum temperature of SHS generated in this study was about 160℃. During these experiments, the SS and SHS temperature was controlled automatically by means of a temperature sensor and solid state relay in each of the steam generators (Fig. 1).

Coupons were removed from the tubes and rinsed as described previously then exposed to SS and SHS on both sides for 5, 10, 15, 20, 25 and 30 s, respectively. SS treatments were performed at 100℃ while SHS treatments were performed at temperatures of 130 and 160℃.

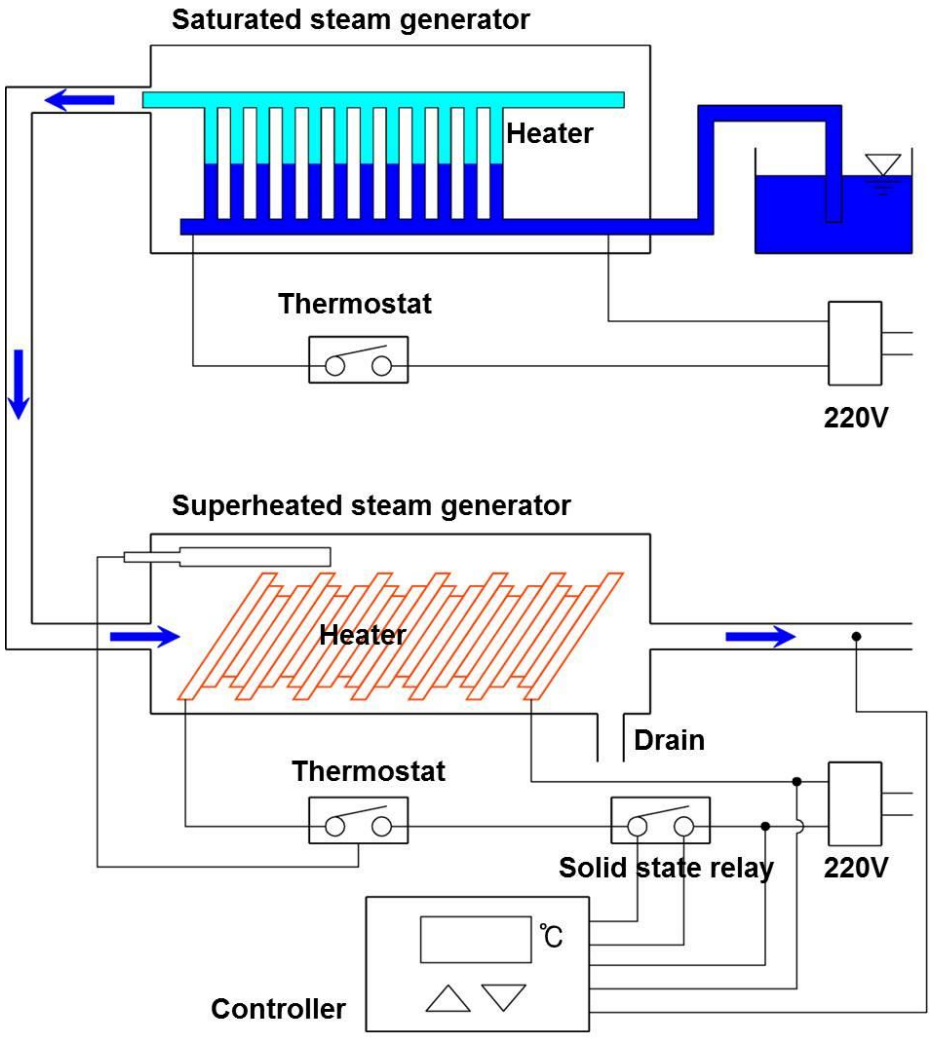


Fig. IV-1. Schematic diagram of the portable superheated steam treatment system

2.4. Bacterial enumeration

After SS and SHS treatment, PVC and stainless steel coupons were deposited in sterile 50-ml conical centrifuge tubes containing 30 ml of PBS and 3 g of sterile glass beads (425–600 μm; Sigma-Aldrich, St. Louis, MO, USA) and then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Cell suspensions in tubes were tenfold serially diluted in buffered peptone water (BPW; Difco) , and then 0.1 ml of undiluted cell suspension or diluents were spread-plated onto Sorbitol MacConkey Agar (SMAC; Difco), Xylose Lysine Desoxycholate Agar (XLD; Difco), and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Difco) to enumerate the number of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* biofilm cells, respectively, attached to the surfaces of PVC and stainless steel coupons. When low bacterial numbers were anticipated, 250 μl of undiluted cell suspension were plated onto four plates of each respective medium. The plates were incubated at 37℃ for 24–48 h and colonies were counted.

2.5. Enumeration of heat-injured cells

To enumerate heat-injured cells of *E. coli* O157:H7, SS and SHS treated coupons were serially diluted and spread-plated onto phenol red agar base with 1% sorbitol (SPRAB; Difco) at time intervals causing the large temperature change (Rocelle et al., 1995). After incubation at 37℃ for 24 h, typical white colonies were enumerated. Random colonies were selected from SPRAB plates and subjected to serological confirmation as *E. coli* O157:H7

(*E. coli* O157:H7 latex agglutination assay; Oxoid), since SPRAB is not a selective media for enumerating *E. coli* O157:H7. The overlay (OV) method was used to enumerate heat-injured cells of *S.* Typhimurium and *L. monocytogenes* using TSA as a nonselective agar and XLD and OAB as the selective agar (Lee and Kang, 2001). Appropriate dilutions were spread-plated onto TSA medium and incubated at 37℃ for 2 h to allow injured cells to recover, and then 7 to 8 ml of XLD or OAB was overlaid on the plates. After solidification, plates were incubated for an additional 22 h at 37℃, and typical black colonies were enumerated.

2.6. Modeling of inactivation kinetics

2.6.1. First-order kinetics

This model assumes a linear relationship between the decline in the logarithm of the number of survivors over treatment time (Schaffner and Labuza, 1997):

$$\log \frac{N}{N_0} = -\frac{t}{D},$$

where N_0 is the initial number of cells (CFU/ml), N the number of survivors after an exposure time t (CFU/coupon), D (decimal reduction time) the time required to destroy 90% of the organisms (min), t the treatment time (min).

2.6.2. Weibull model

The Weibull model assumes that cells and spores in a population have different resistances, and a survival curve is just the cumulative form of a

distribution of lethal agents. The cumulative form of the Weibull distribution is as follows:

$$\log(N) = \log(N(0)) - (\frac{t}{\delta})^{p}$$

where δ and p are the scale and shape factors (Peleg and Cole, 1998), respectively.

2.7. Statistical analysis

All experiments were repeated three times with duplicate samples. Data was analyzed by analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, Cary, NC, USA) and the separation of means was tested by Duncan's multiple range tests at a probability level of $P < 0.05$.

IV-3. Results and Discussion

Fig. 2 shows survival of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* biofilm cells on stainless steel after SS and SHS steam treatment. The initial level of them was 7.07, 7.15, and 6.67 log CFU/coupon, respectively. Slight reductions (< 0.5 log) occurred when inoculated samples were rinsed in water for controls (data not shown). This phenomenon was mainly attributable to the physical removal of biofilm cells from stainless steel coupons during washing.

The levels of *E. coli* O157:H7 biofilm cells survived were significantly ($P < 0.05$) reduced as the duration of SHS treatment increased. *E. coli* O157:H7 biofilm cells experienced a log reduction range of 0.69–3.25 after SS treatment at 100℃ for 5–30 s, but were reduced by 0.5–5.59 log to below the detection limit (1.48 log CFU/coupon), after SHS treatment at 160℃ for the same time. SS treatment 100℃ for 5–30 s brought about 0.3 to 3.46 log decreases in *S.* Typhimurium biofilm cells, whereas SHS treatment 160℃ reduced bacterial cell numbers below the detection limit, showing 0.57–5.67 log decrease for 5–30 s. SS treatment resulted in 0.32–2.95 log reductions in surviving *L. monocytogenes* biofilm cells, while SHS treatment caused 0.96–5.19 log reduction for the equivalent times. It was observed that the SHS treatment caused an additional log reduction compared to the SS treatments. The populations of viable biofilm cells on stainless steel coupons were

reduced below the detection limit when subjected to SHS treatment at 160℃ for 30 s.

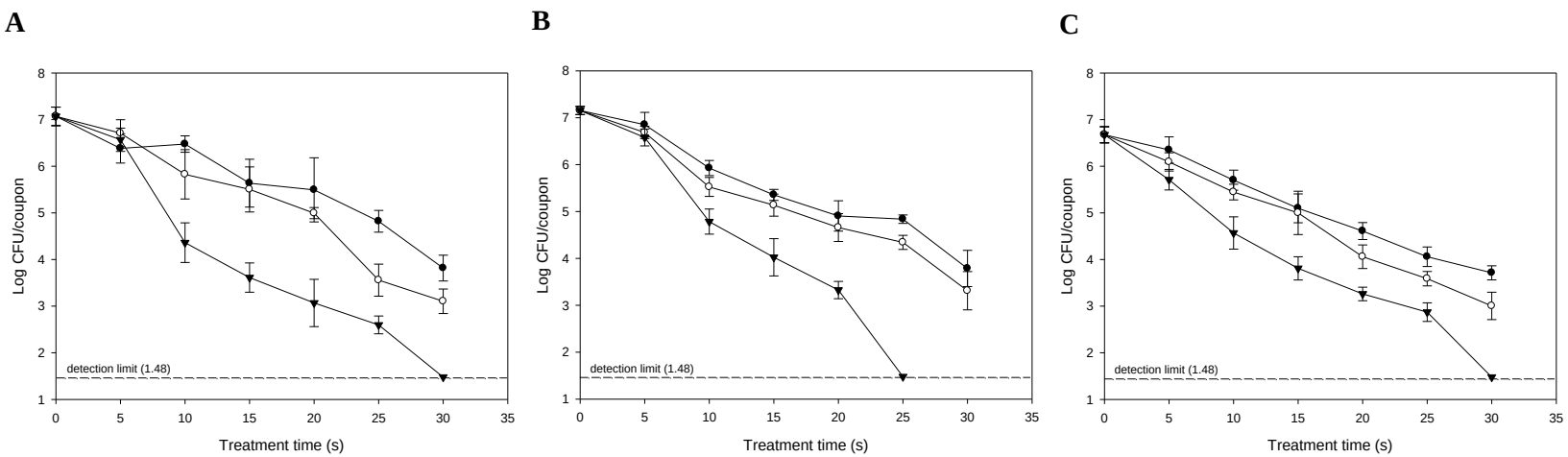


Fig IV-2. Survival (log CFU/coupon) of *E. coli* O157:H7 (A), *S.* Typhimurium (B), *L. monocytogenes* (C) in biofilm formed on the surface of stainless steel coupons treated with saturated steam and superheated steam.

Surviving cells and heat-injured cells of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on stainless steel coupons following SS or SHS heating were compared (Table 1). There were no significant ($P > 0.05$) differences between the levels of cells enumerated on the appropriate selective agar (SMAC, XLD, and OAB) versus the agar for resuscitation (SPRAB, OV-XLD, and OV-OAB) during the whole treatment time. However, SS heating 100℃, statistically significant ($P < 0.05$) differences between levels of surviving cells and cells including sub-lethally injured *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were observed after 25 and 30 s, 30 s, 15, 20, 25, and 30 s treatments, respectively.

Table IV-1. Survival (log CFU/coupon) of uninjured cells and injured *E. coli* O157:H7 (A), *S.* Typhimurium (B), *L. monocytogenes* (C) in biofilm for med on the surface of stainless steel coupons treated with saturated steam and superheated steam

(A)	Population (log CFU/coupon)					
	100℃		130℃		160℃	
Treatment time (s)	SMAC	SPRAB	SMAC	SPRAB	SMAC	SPRAB
0	7.07±0.20Aa	6.87±0.31Aa	7.07±0.20Aa	6.87±0.31Aa	7.07±0.20Aa	6.87±0.31Aa
5	6.38±0.31Ba	6.55±0.22Aa	6.71±0.29Aa	6.53±0.42Aa	6.57±0.25Ba	6.44±0.13Aa
10	6.48±0.18Ba	6.31±0.29Aba	5.83±0.53Ba	6.01±0.29Ba	4.36±0.42Ca	4.75±0.33Ba
15	5.64±0.51BCa	5.92±0.12Ba	5.50±0.48BCa	5.47±0.24BCa	3.61±0.31Da	3.86±0.29Ca
20	5.49±0.69BCa	5.86±0.41BCa	4.99±0.12Ca	5.19±0.22Ca	3.07±0.51DEa	3.21±0.41CDa
25	4.82±0.23Ca	5.43±0.26Cb	3.56±0.34Da	3.76±0.32Da	2.60±0.19Ea	2.79±0.38Da
30	3.82±0.28Da	4.47±0.29Db	3.11±0.26Da	3.48±0.31Da	< 1.48Fa	< 1.48Ea

(B)	Population (log CFU/coupon)					
	100℃		130℃		160℃	
Treatment time (s)	XLD	OV-XLD	XLD	OV-XLD	XLD	OV-XLD
0	7.15±0.09Aa	6.86±0.14Aa	7.15±0.09Aa	6.86±0.14Aa	7.15±0.09Aa	6.86±0.14Aa
5	6.85±0.31Aa	6.52±0.69ABa	6.69±0.13Ba	6.64±0.27Aa	6.58±0.18Ba	6.51±0.22Aa
10	5.93±0.18Ba	6.13±0.22Ba	5.52±0.20Ca	5.77±0.48Ba	4.79±0.27Ca	5.23±0.52Ba
15	5.35±0.51BCa	5.66±0.53BCa	5.14±0.24Ca	5.21±0.31BCa	4.02±0.40Da	4.42±0.18Ca
20	4.91±0.69Ca	5.24±0.36Ca	4.66±0.30Da	4.85±0.27Ca	2.83±0.33Ea	3.19±0.41Da
25	4.84±0.23Ca	4.74±0.17CDa	3.88±0.15Ea	4.01±0.29Da	< 1.48Fa	< 1.48Ea
30	3.79±0.28Da	4.51±0.31Db	3.31±0.41Ea	3.61±0.38Da	< 1.48Fa	< 1.48Ea

(C)	Population (log CFU/coupon)					
	100℃		130℃		160℃	
Treatment time (s)	OAB	OV-OAB	OAB	OV-OAB	OAB	OV-OAB
0	6.67±0.17Aa	6.63±0.31Aa	6.67±0.17Aa	6.63±0.31Aa	6.67±0.17Aa	6.63±0.31Aa
5	6.35±0.28Aa	6.41±0.19Aa	6.09±0.20Ba	6.28±0.27Aba	5.71±0.22Ba	5.52±0.33Ba
10	5.70±0.22Ba	6.02±0.31ABa	5.45±0.17Ca	5.72±0.39Ba	4.57±0.35Ca	4.58±0.16Ca
15	5.10±0.31Ca	5.77±0.22Bb	4.90±0.23Da	5.03±0.28Ca	3.81±0.25Da	4.02±0.11Da
20	4.61±0.18Ca	5.26±0.39BCb	4.06±0.25Ea	4.31±0.17Da	3.26±0.15Ea	3.38±0.19Ea
25	4.06±0.21Da	4.69±0.28Cb	3.59±0.15Fa	3.71±0.22Ea	2.87±0.20Fa	2.96±0.21Fa
30	3.72±0.15Da	4.21±0.30Cb	3.01±0.29Ga	3.50±0.17Eb	< 1.48Ga	< 1.48Ga

Mean of three replications ± standard deviation.
Means with the same capital letter in the same column are not significantly different ($P > 0.05$).
Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
XLD, Xylose Lysine Desoxycholate; OV-XLD, overlay XLD agar on TSA; SMAC, Sorbitol MacConkey agar; SPRAB, Phenol red agar base with 1% sorbitol; OAB, Oxford Agar Base; OV-OAB, overlay OAB agar on TSA.

The Weibull and traditional linear model for the inactivation of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* biofilm cells on stainless steel at different SS and SHS heating temperature and treatment time is represented Table 2 and 3. The goodness of fit of the linear and Weibull models were compared by calculating the R^2 and RMSE values. Comparing the individual and mean values, it could be postulated that the linear model produced poorer fit to the results data as indicated by its lower R^2 and higher RMSE values when compared to the Weibull distribution. However, some researchers criticized using R^2 as it has little significance when the model form is nonlinear (Davey, 1993). For this reason, further a correlation plot was drawn for judging the goodness of fit of these models. When the residual and correlation plots of these two models analyze, differences of applicability of the models exposed. These results indicate that there is a close relationship between observed and fitted values for Weibull model, but not for the linear first-order model.

It seems logical to use Weibull distribution because microbiologists generally use logarithms of numbers for obvious reasons. There is, of course, a statistical pitfall because a transformation changes the error structure of data and this may disturb the subsequent regression. However, with microbiological counts, the logarithmic transformation induces a normal distribution of errors and stabilizes the variance (van Boekel, 2002).

Shape factors (p) of Weibull model in Table 3 indicate that survival curves of the three pathogens treated with SS and SHS fitted with this model were

concave upward ($p < 1$). Although the Weibull model is of an empirical nature, a link can be made with microbial inactivation as follows (Buzrul and Alpas, 2007). Upward concavity ($p < 1$) indicates that remaining members have the ability to adapt to applied stress; whereas downward concavity ($p > 1$) indicates that remaining members become increasingly damaged (van Boekel, 2002). Therefore, concave upward ($p < 1$) survival curves of these three pathogens fitted with this model can be interpreted as evidence that weak or sensitive members of the populations are eliminated at a relatively fast rate leaving behind survivors of higher and higher resistance (Buzrul et al., 2005). In literature, both concave-downward and concave-upward survival curves were fitted with the Weibull model.

In this study, our aim was to present an example of how the Weibull model can be implemented in the analysis of three pathogens biofilms inactivation by SS and SHS. The Weibull model produced better fit to the data than the traditional linear model for all of the treatments. Accurate model prediction of survival curves would be beneficial to the food industry in selecting the optimum conditions of temperature and time to obtain desired levels of inactivation of *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* biofilm cells on stainless steel while minimizing the production costs. Our results have revealed that the Weibull model, which had been mostly used for describing inactivation of the bacterial cells by heat treatment, could be successfully used to describe foodborne pathogens biofilm cells on stainless steel inactivation by SHS.

Table IV-2. Evaluation first order model for the survival curves of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilm cells on stainless steel treated with SS and SHS

	Treatment temperature (℃)	MSE	R^2
E. coli O157:H7	100	0.74	0.92
	130	0.30	0.94
	160	1.13	0.89
S. Typhimurium	100	0.23	0.94
	130	0.17	0.92
	160	1.41	0.88
L. monocytogenes	100	0.39	0.94
	130	0.37	0.95
	160	0.98	0.91

MSE, mean square error; R^2, regression coefficient.

Table IV-3. Evaluation Weibull model for the survival curves of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilm cells on stainless steel treated with SS and SHS

	Treatment temperature (℃)	δ	p	MSE	R^2
E. coli O157:H7	100	14.96	1.54	0.07	0.96
	130	10.27	1.30	0.07	0.98
	160	3.35	0.79	0.24	0.96
S. Typhimurium	100	8.61	0.95	0.07	0.97
	130	6.88	0.92	0.04	0.99
	160	5.11	1.10	0.08	0.99
L. monocytogenes	100	9.53	0.99	0.01	0.99
	130	7.99	0.99	0.01	0.99
	160	4.61	0.85	0.08	0.98

MSE, mean square error; R^2, regression coefficient.

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국문초록

과열수증기는 주어진 압력에서 끓는 점 이상으로 가열된 수증기를 말한다. 과열수증기는 안전하고 에너지가 상대적으로 적게 소비되는 무공해 기술로써 여러 가지 물질의 건조에 이용되는 효과적인 방법중의 하나로 알려져 오고 있다. 그러나 과열수증기를 이용한 식품병원성균 저감화에 관한 연구는 거의 진행되지 않았다. 본 연구에서는 과열수증기를 이용하여 *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* 로 대표되는 식품병원성균의 저감화 효과에 대한 연구를 진행하였다.

세균은 식품공정기기 표면에 부착할 수 있고, 세포외 중합체으로 구성된 미끈한 바이오필름 (생물막)을 형성할 수 있다. 식품공정 기기 표면에의 세균 부착은 식품으로의 오염을 야기하므로 위생상 큰 문제 위험이 있다. *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* 바이오필름을 PVC와 스테인리스 쿠폰 위에 형성시킨 후, 100℃의 포화수증기와 125, 150, 175, 200℃의 과열수증기로 5, 10, 20, 30 초 처리하였다. 과열수증기 온도와 처리 시간이 증가할수록 바이오필름 균의 수가 유의적으로 감소하였으며, 포화수증기로 처리했을 경우보다 추가적인 저감화를 확인할 수 있었다. 특히, 과열수증기 200℃로 PVC에 30초, 스테인리스에 10초 처리시, 바이오필름 균의 수가 검

출한계 (1.48 log CFU/coupon) 아래로 감소하였다.

과열수증기를 이용한 농산물 병원성균 저감화효과를 확인하기 위하여, 아몬드, 피스타치오, 방울토마토, 오렌지, 무종자, 알팔파 종자표면의 식품병원성균 저감화효과를 알아보고 열처리에 따른 식품의 상태 변화를 관찰하였다. 과열수증기 200℃로 아몬드는 15초, 피스타치오는 30초 처리했을 경우, 색깔과 질감의 유의적인 변화없이 *E. coli* O157:H7, *S.* Typhimurium, *S*. Enteritidis PT 30, *L. monocytogenes* 가 검출한계 (0.3 log CFU/g) 아래로 떨어졌으며, 산가와 과산화물가는 허용범위 내로 측정되었다. 과열수증기 200℃로 방울토마토 3초, 오렌지 20초 처리했을 경우, 색깔, 질감, 아스코르브산 함량, 항산화력의 유의적인 변화없이 *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* 가 검출한계 (1 또는 1.7 log CFU/g) 아래로 떨어졌다. 종자 살균에서는 연속적인 스팀처리로 무종자는 3초, 알팔파는 2초 안에 발아율이 상당히 감소하였다. 그러나, 1초 스팀처리 후 2분 상온 냉각하는 방법은 10반복 처리에도 발아율을 95% 유지하였으며 과열수증기로 처리시 3 log 이상의 저감화를 나타내었다.

전산유체역학를 이용하여 과열수증기 처리 동안 오렌지표면의 *Salmonella*의 저감화 양상을 시뮬레이션하였다. 컴솔 다중물리 소프트웨어를 이용하여 오렌지 표면의 생균수와 챔버내의 온도분포를 예측하였다. 연속성, 압축성 유체, 에너지 지배방정식은 박테리아 수

와 함께 유한요소법으로 수치계산이 되었으며, 박테리아 저감화는 아레니우스 식으로 나타내었다. 시뮬레이션 결과는 과열수증기 처리 시간에 따른 챔버 내 유체의 흐름, 온도분포, 박테리아 저감화 양상을 보여주었다. 또한, 결과를 통해 과열수증기 처리 이용 시 과열수증기가 빠르게 챔버 내로 분포하여 균저감화 또한 급속도로 일어난다는 것을 확인하였다.

과열수증기 시스템을 실제 현장에 적용하고자 운반이 용이한 사이즈의 발생기를 자체 구축하였고, 효과 평가를 위해 병원균 바이오필름 저감화를 통해 확인해보았다. 그 결과, 160℃ 과열수증기로 30초 처리시 스테인리스의 바이오필름의 수가 검출한계 아래로 감소하였으며, 상처 입은 박테리아와 유의적인 차이를 나타내지 않았다. 또한, 산업에의 적용을 위해 일차식과 웨이불 식을 이용하여 적합성을 R^2 를 통해 측정한 결과, 웨이불 모델이 적합함을 확인하였다.

본 연구에서는 과열수증기의 바이오필름과 농산물 표면의 병원균 제어능력에 대한 뛰어난 효과를 포화수증기와의 비교를 통해 증명하였다. 또한 실제 현장에 적용을 위해 전산유체역학을 통해 균저감화 양상을 확인하였고, 휴대용 과열수증기 발생기를 제작하여 균 저감화 효과를 확인하였다. 종합적으로, 과열수증기 기술은 식품산업에서 식품병원균을 빠르고 효과적으로 제어할 수 있음을 확인하였다.

핵심어: 과열수증기, 장출혈성 대장균 O157:H7(*Escherichia coli* O157:H7), 살모넬라 티피뮤리움 (*Salmonella* Typhimurium), 리스테리아 모노사이토제니스 (*Listeria monocytogenes*), 바이오필름, 전산유체역학, 휴대용